The role of *Fam20a* in the generation of Amelogenesis Imperfecta

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät der

Universität Zürich

von

Anna Filatova

aus

Russland

Promotionskomitee

Prof. Dr. Michael Hengartner

Prof. Dr. Thimios Mitsiadis

Prof. Dr. Sabine Werner

Prof. Dr. Freddy Radtke

Prof. Dr. Alex Hajnal

Zürich, 2015

Table of Contents

1. Summary

Tooth development is a complex process involving highly organized cellular and molecular mechanisms. Defects in the development of any component of teeth may lead to functional dental defects. Defects in enamel structure and appearance caused by genetic mutations are indicated by the term Amelogenesis Imperfecta (AI). Despite recent important findings in the field of AI a lot of key questions remain unanswered.

The recently discovered family with sequence similarity 20 (Fam20) received a lot of attention. Members of this family are the Fam20a, Fam20b, and Fam20c molecules. These molecules are predicted to be secreted protein kinases involved in biomineralization processes. While mutations in *Fam20a* have been shown to lead to AI the exact role of *Fam20a* in amelogenesis is not well understood. In order to shed light on the involvement of this kinase in odontogenesis and most particularly in amelogenesis we first analyzed the expression of the *Fam20a* gene and its protein in developing teeth. Our results showed that *Fam20a* is specifically expressed in dental tissues. Additionally, we found that Fam20a protein expression does not always follow *Fam20a* gene expression. To evaluate the roles of *Fam20a* in odontogenesis, we used a transgenic mouse model in which the first exon and part of the first intron of the *Fam20a* gene were replaced with the LacZ cassette. Histological analyses and micro-computed tomography showed that *Fam20a*$^{-/-}$ mice form disorganized layers of mineralized material instead of well-structured and fully mineralized enamel. This defective enamel disintegrates shortly after tooth eruption, leading to the destruction of the underlying dentin. Furthermore, we have observed that the enamel-producing ameloblastic cells detach from the predentin/dentin after reaching their secretory stage, loose their polarity and orientation and this disorganization has as consequence the formation of enamel anomalies. Since tooth development depends on epithelial/mesenchymal interactions we have evaluated the role of these interactions on Fam20a expression using tissue recombination experiments. The experiments have clearly demonstrated that enamel defects in *Fam20a*$^{-/-}$ mice are caused exclusively from an epithelial defect and this cannot be rescue from with signaling from a wildtype mesenchyme. Therefore, we tested if exogenous FAM20A protein could rescue the enamel phenotype observed in the *Fam20a*$^{-/-}$ mice. We showed that exogenous FAM20A protein penetrates into developing tooth germs and that ameloblasts of the formed teeth are polarized and attached to the extracellular matrix.

Taken together, the present results show a critical and specific role of Fam20a protein in enamel formation and suggest new treatment strategies for specific forms of AI.

2. Zusammenfassung

Die Entwicklung von Zähnen ist ein komplexer Prozess, der auf vielen hochgradig organisierten zellulären und molekularen Mechanismen basiert. Fehler in der Entwicklung einer der Zahnkomponenten können die volle Funktionalität der Zähne beeinträchtigen. Aus genetischen Mutationen resultierende Zahnschmelzdefekte werden unter dem Begriff Amelogenesis Imperfecta (AI) zusammengefasst. Trotz wichtiger Erkenntnisse auf dem Gebiet der AI Forschung, gibt es noch viele unbeantwortete Schlüsselfragen.

Die vor Kurzem entdeckte Familie mit Sequenzähnlichkeit 20 (Fam20) hat in diesem Zusammenhang viel Aufmerksamkeit bekommen. Innerhalb dieser Familie werden die Moleküle Fam20a, Fam20b und Fam20c unterschieden und es wird vermutet, dass es sich bei diesen Molekülen um sekretierte Proteinkinasen, die in Biomineralisationsprozesse involviert sind, handelt. Während für Fam20a gezeigt werden konnte, dass Mutationen zu AI führen, ist die Rolle, die Fam20a in der Amelogenese spielt bis heute nicht genau bekannt. Um die Beteiligung dieser Kinase an der Odontogenese und vor allem an der Amelogenese besser zu verstehen, haben wir zunächst die Expression des Fam20a Gens und dessen Protein in sich entwickelnden Zähnen untersucht. Unsere Versuche ergaben, dass Fam20a spezifisch in dentalen Geweben exprimiert wird. Um die Funktion von Fam20a während der Odontogenese zu untersuchen, wurde ein transgenes Mausmodell verwendet, in dem das erste Exon und ein Teil des ersten Introns des Fam20a Gens durch eine LacZ Kassette ersetzt wurden. Anhand von histologischen Analysen und einer Auswertung mittels Mikro-Computertomographie konnte gezeigt werden, dass *Fam20a*$^{-/-}$ Mäuse Schichten von desorganisiertem mineralisierten Material bilden statt eines gut strukturierten vollständig mineralisierten Zahnschmelzes. Dieser defekte Zahnschmelz in *Fam20a*$^{-/-}$ Mäusen zerfällt kurz nach Zahndurchbruch, sodass das darunterliegende Dentin ungeschützt weiterer Zerstörung ausgesetzt ist. Des Weiteren konnten wir beobachten, dass die Zahnschmelz-produzierenden Ameloblasten sich vom Predentin/Dentin ablösen sobald sie die sekretorische Phase erreicht haben und auch ihre Polarität und Ausrichtung verlieren, was zusammen zu den genannten Zahnschmelzanomalien führt. Da die Entstehung von Zähnen von epithelialen/mesenchymalen Interaktionen abhängt, haben wir anhand eines Rekombinationsexperiments analysiert, welchen Einfluss Fam20a auf diese Interaktionen hat. Die Versuche haben eindeutig gezeigt, dass Defekte im Zahnschmelz von *Fam20a*$^{-/-}$ Mäusen ausschliesslich von einer Fehlfunktion des Epithels verursacht werden und nicht durch ein funktionelles Mesenchym korrigiert werden können. Daher wurde getestet, ob der in *Fam20a*$^{-/-}$ Mäusen beobachtete Zahnschmelzdefekt durch Zugabe von exogenem FAM20A Protein repariert werden kann.

Zusammengenommen weisen die gesammelten Ergebnisse auf eine entscheidende und auch spezifische Funktion des Fam20a Proteins bei der Bildung von Zahnschmelz auf und könnten zu der Entwicklung neuer Behandlungsmethoden bei bestimmten Formen von AI beitragen.

3. Introduction

3.1 Teeth

The main function of teeth is linked to the mastication process. Although, being highly specialized organs, teeth are optimal tools for many fields of research, such as evolutionary, developmental, and molecular biology. The tooth model allows studies on epithelial mesenchymal interactions, mineralization processes, innervation, and vascularization (Pagella et al. 2014; Mitsiadis et al. 2014; Filatova et al. 2014). Due to their highly mineralized structure, teeth can be stored for many years without losing shape (Weil 2003). The pattern of teeth provides information about nutrition and thus feeding adaptations and animal behaviour (Tucker & Sharpe 2004).

To date, there are two competing hypotheses on the evolutionary origin of teeth. The first "outside-in" hypothesis claims that teeth are originated from external tooth-like dermal denticles that comprise fish scales. In the vertebrate phylogeny teeth moved to the oral cavity and adapted to their mastication function. The second, "inside-out" hypothesis, states that teeth evolved independent of other tooth-like structures located outside the mouth (Donoghue & Rücklin 2014).

There are different types of human teeth, which are incisors, canines, premolars, and molars, but their basic structure and components are quiet similar (Fig. 3.1). The tooth is divided into two parts: the visible part is the crown and the hidden part is formed by the roots. The tooth is constructed by both mineralized and soft tissues. Three types of mineralized tissues can be distinguished: the enamel, the dentin and the cementum. The enamel is the hardest tissue of the body and covers the crown of the tooth. It is the only epithelial-derived calcified tissue in vertebrates (Bartlett 2013a). The dentin is localized under the enamel in the crown and under the cementum in the roots and is comprised by 20-30% of organic material by weight. It is characterized by both hardness and elasticity. This combination provides flexibility of the tooth and prevents enamel damage (Bartlett 2013a). The cementum covers the root of the tooth. Periodontal ligament (PDL) attaches to the cementum of the roots. The PDL, alveolar bone, and cementum form the periodontium, which provides fixation of the tooth to the jaw (Fleischmannova et al. 2010; Diep et al. 2009; Catón et al. 2011). Dental pulp is the soft connective tissue which occupies the inner part of the crown and the roots, called pulp chamber.

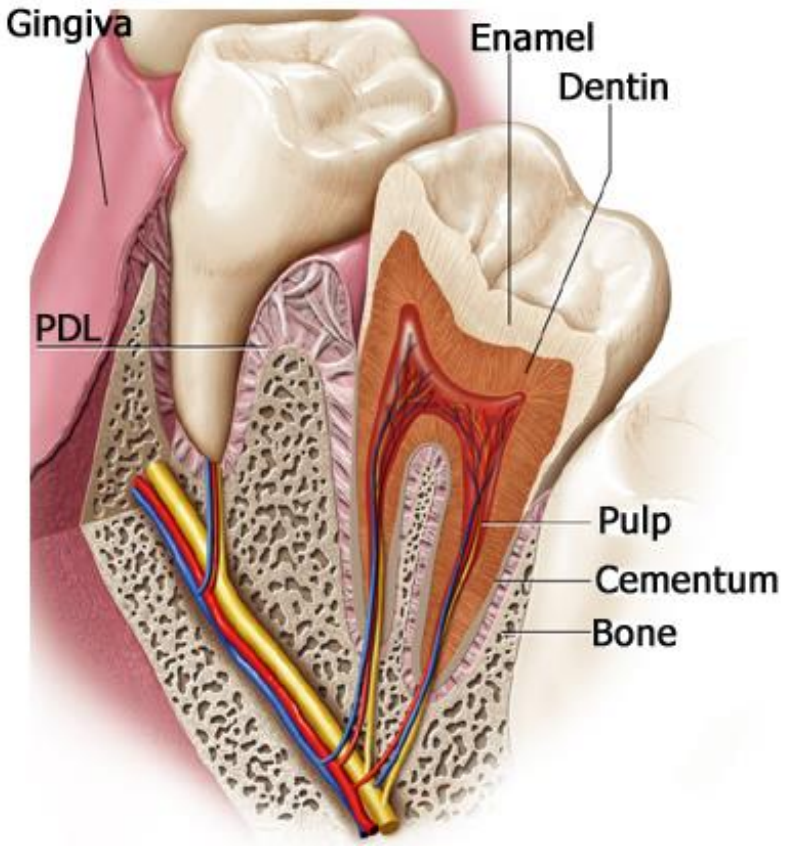


***Figure 3.1**. Anatomy of the dental structures. PDL – periodontal ligament (Nanci 2003).*

The formation and patterning of teeth in mice and human share great similarity, especially at the earliest developmental stages (Zhang et al. 2005). Both mice and human teeth go through all the main stages of odontogenesis. Mouse dentition is considered to be a simplified model of human dentition (Tucker & Sharpe 2004). Mice have only two types of teeth: the molars and the incisors (Fig. 3.2). The structure and the development of mouse molars are similar with those of the human teeth. However, mouse incisors present significant differences. The incisor is a continuously growing organ that erupts throughout the animal's life, has a cylindrycal shape and is formed by two distinct epithelia that surround the dental pulp: the lingual and the labial epithelia. The lingual side of the incisor is considered as a root analogue of the molar characterized by the deposition of cementum, while the labial side is the crown analogue of molars characterized by the deposition of enamel. The incisor is divided into the anterior erupting part and the posterior part, where the niches of epithelial and mesenchymal stem cells reside. The posterior part of the incisor containing the epithelial stem cells is called cervical loop (CL) area. Cells from the CL proliferate and migrate towards the anterior part and differentiate into enamel producing ameloblasts (Mitsiadis & Graf 2009).

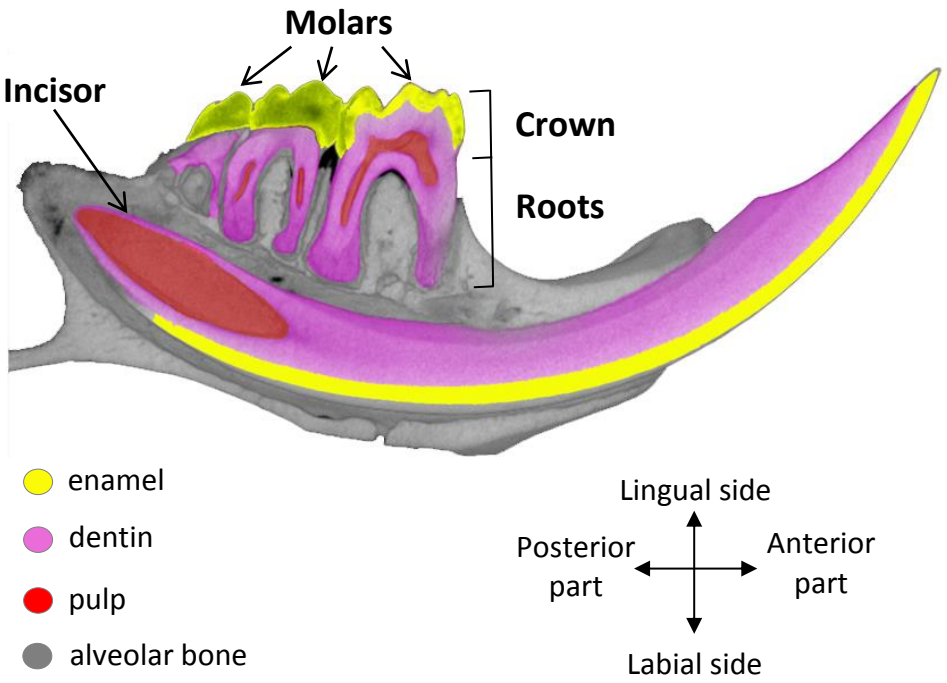


***Figure 3.2**. Mouse teeth.*

The various stages of tooth development

Tooth development depends on two tissues: the oral epithelium and the cranial neural crest-derived mesenchyme. Odontogenesis proceedes through well defined stages, which are the stages of dental lamina, dental placode, bud, cap, bell, mineralization, and the eruption stage (Fig. 3.3) (Jussila & Thesleff 2015).

Tooth initiation starts at embryonic day 10.5 (E10.5) in mice and approximately at 5-6 weeks of embryonic development in humans (Mitsiadis & Graf 2009; Tucker & Sharpe 2004). *The dental lamina* is the first sign of tooth formation. It is followed by the thickening of the oral epithelium that gives rise to the dental *placode*.

The epithelium continues to grow and invaginates into the underlying mesenchyme forming the *bud*. The mesenchyme condenses around the bud epithelium and progressively becomes the dental mesenchyme (Mitsiadis & Graf 2009; Tucker & Sharpe 2004). Thereafter, the mesenchyme divides into two parts: one which gives rise to the dental pulp and dentin-producing odontoblasts, and the other to the dental follicle which will form the periodontal tissues (Nanci 2003).

Continuous extension of epithelial cells brings the tooth to the *cap* and *bell* stages (Mitsiadis & Graf 2009; Tucker & Sharpe 2004). At these stages, the epithelium forms the enamel organ and consists of distinct cell layers: inner enamel epithelium, outer enamel epithelium, stellate reticulum, and stratum intermedium. Also the main signalling center of the tooth called the enamel knot is

formed, which is necessary for tooth morphology. Secondary enamel knots are formed at the bell stage. The enamel knots determine the crown morphology of the teeth (Matalova et al. 2005).

At the bell stage, cells from the pulp adjacent to the dental epithelium differentiate into odontoblasts and start to produce dentin. The function of the pulp is to provide vitality to the tooth and to respond to bacterial invasions, injury, and mechanical stimuli. Histologically the dental pulp can be divided into four zones: odontoblastic zone, cell-free zone (of Weil), cell-rich zone, and the pulp core. Dental pulp is made by several cell types: odontoblasts, fibroblasts, undifferentiated ectomesenchymal cells, macrophages, and other immunocompetent cells. At the apical part of the tooth nerves and blood vessels enter into the tooth pulp where they form an extensive vascular and nervous network (Nanci 2003). The dental pulp contains pools of stem cells that are activated upon dental injury (Ravindran & George 2015; Mitsiadis et al. 2011). At the same time, epithelial cells next to the dental pulp differentiate into ameloblasts and start to form enamel (Mitsiadis & Graf 2009). As soon as deposition of the mineralized matrices has been completed and the tooth shape is fixed, tooth eruption starts followed by root growth (Jussila & Thesleff 2015).

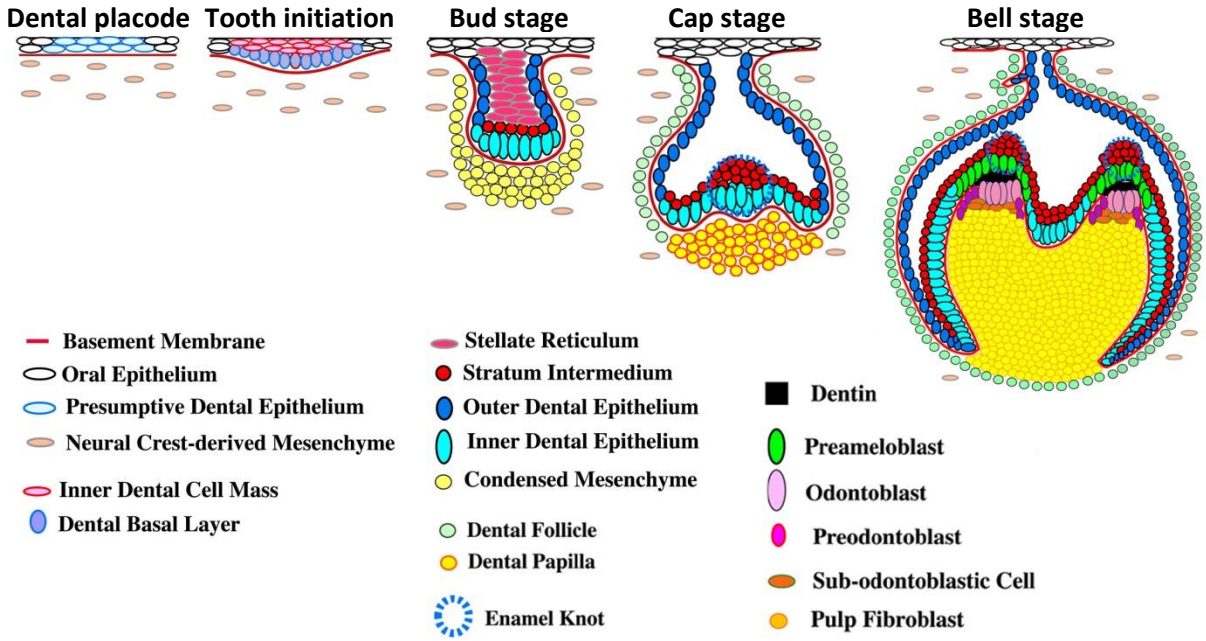


Figure 3.3. *Stages of tooth development* (Mitsiadis & Graf 2009).

Numerous studies have shown that a plethora of signaling pathways, transcription factors, and extracellular matrix molecules are involved in all stages of tooth development. The main signaling molecules belong to five well-known signaling pathways: Notch, bone morphogenetic protein (Bmp), fibroblast growth factor (Fgf), sonic hedgehog (Shh), and wingless/integration 1 (Wnt) (Mitsiadis & Luder 2011). These molecules can mediate cell communication within one tissue or be involved in

the cross-talk between tissues. Control of tooth formation by signaling molecules occurs via coordination of cell proliferation, differentiation, apoptosis, extracellular matrix synthesis, and mineral deposition (Mitsiadis & Graf 2009).

The expression of some of these molecules is crucial and their deletion may lead to partial or complete loss of the dentition. For example, Bmp7-deficient mice often lack incisors and in some cases also molars. Loss of Ectodysplasin (Eda) leads to a reduction of a number of teeth while overexpression of Eda leads to the formation of supernumerary teeth. Complete arrest of tooth development is observed after mutations of transcription factors such as Lef1, Msx1, Pitx2, and Pax9 (Cobourne & Sharpe 2010; Mitsiadis & Luder 2011; Mitsiadis & Graf 2009).

Cytodifferentiation and mineralization events during odontogenesis

During this stage of odontogenesis the dental tissues are specialized and the mineralization process is active. Initiation of enamel and dentin formation depends on the reciprocal induction of odontoblasts and ameloblasts (Fig. 3.4). The differentiation of odontoblasts precursors into odontoblasts is initiated by cells of the inner enamel epithelium. This guidance is provided by signalling molecules and growth factors released by cells of the inner enamel epithelium. Early differentiation of odontoblasts is accomplished by the secretion of the organic matrix of dentin. The organic matrix is deposited by odontoblast-cytoplasmic extensions. Dentin forms around these extensions, thus leading to the formation of a tubular structure. Preameloblasts differentiate into ameloblasts and secret the first enamel layer against the newly formed dentin (Nanci 2003).

The secretory odontoblasts and ameloblasts are shown in Figure 3.5. Odontoblasts are highly polarized columnar cells (50-60µm). They have two portions: the odontoblastic body and the odontoblastic process. The organelles are localized in the odontoblastic body, while the odontoblastic extension represents the secretory part of the cell containing secretory granules with predentin and dentin components. Ameloblasts are also columnar highly polarized cells (50-70µm in height). The nucleus is localized in the basal part, while in the apical (secretory) part the ameloblast forms the Tomes' process, which contains secretion granules and free polysomes (Ruch, 1987).

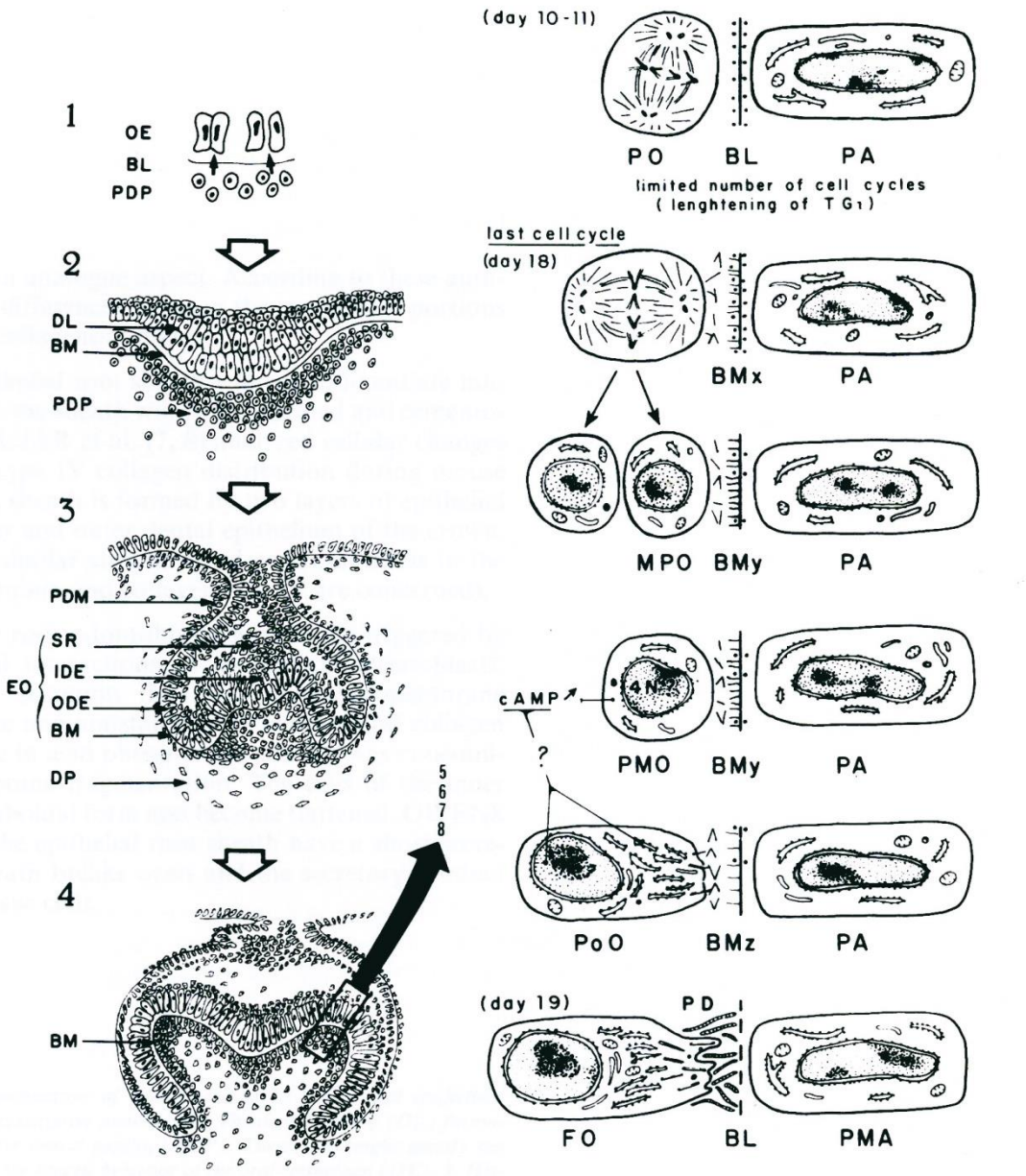


Figure 3.4. *Reciprocal interaction of odontoblast and amelosblasts during differentiation. BL: basal lamina; BM: basement membrane; DL: dental lamina; DP: dental papilla; EO: enamel organ; IDE: inner dental epithelium; MP: mature preodontoblasts; ODE: outer dental epithelium; OE: oral epithelium; PA: preameloblasts; PDM: peridental mesenchyme; PDP: presumptive dental papilla; PMO: post-mitotic odontoblasts; SR: stellyte reticulum (adapted from Ruch, 1987).*

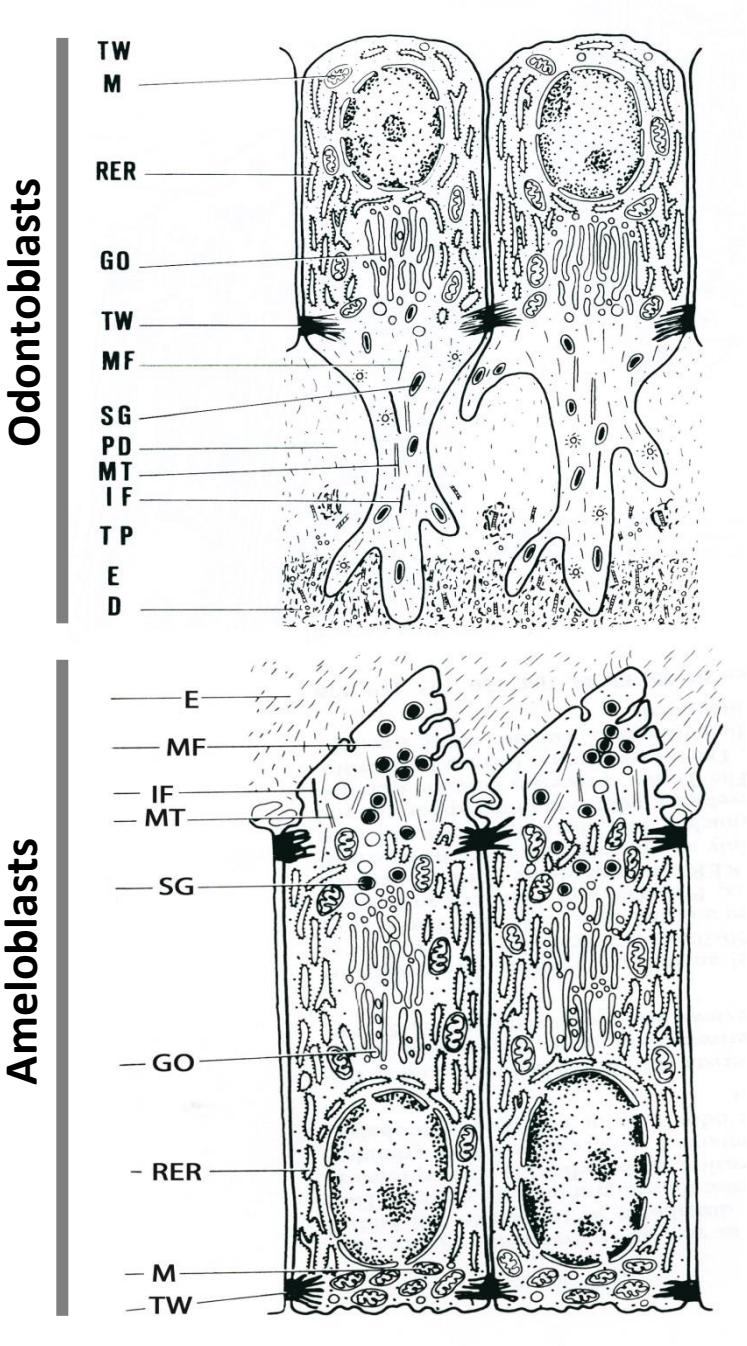


Figure 3.5. *Structure of functional odontoblasts and ameloblasts and their mutual arrangement: D: dentin; E: enamel; GO: Golgi apparatus; IF: intermediate filaments; M: mitochondria; MF: microfilaments; MT: microtubules; PD: predentin; RER: rough endoplasmic reticulum; SG: secretory granule; TM: terminal web; TP: Tomes' process; TW: terminal web (adapted from Ruch, 1987).*

Dentinogenesis

Dentin is the second hardest tissue in the body, composed by 70% inorganic material, 20% organic material, and 10% water by weight. The formation of dentin begins at the bell stage of tooth development. At this stage odontoblast precursors appear as small cells with a centrally located nucleus that progressively enlarge and elongate forming the layer of preodontoblasts. Further differentiation to odontoblasts is accompanied by increasing cell volume, protein synthesis, and polarization (Fig. 3.5). The first sign of dentin formation is the production of large fibrils of collagen III, then the production of smaller collagen I fibrils. Mineralization of dentin is achieved by continuous deposition of mineral, a process that is regulated by proteins produced by odontoblasts (Nanci 2003).

The dentin comprise both collagen and non-collagenous matrix proteins. The non-collagenous matrix proteins include Dentinsialophosphoprotein (Dspp), Dentin matrix proteins-1, -2, and -3 (Dmp-1, Dmp-2, Dmp-3) and small amounts of proteins that are also found in bone: Osteopontin (Osp), Bone sialoprotein (Bsp), Osteonectin (Osn), and Osteocalcin (Osc) (Arana-Chavez, 2004).

Amelogenesis

Enamel is the most mineralized tissue of the body since its mineral content reaches 98%, the other 2% being organic molecules and water (Gordon & Joester 2015). The enamel has strong mechanical properties such as toughness and wear resistance that support tooth functions during mastication. These properties are reached by the organization of hydroxylapatite crystals in enamel rods (Fig. 3.6) (Gordon & Joester 2015). The space between the rods is occupied by the interrod enamel, which is also formed by crystallites. The difference between the rod and the interrod enamel lies mainly in the orientation of the crystallites, but also the interrod enamel is more organic-rich compared to the rod enamel (Hu et al. 2007; Ang et al. 2012).

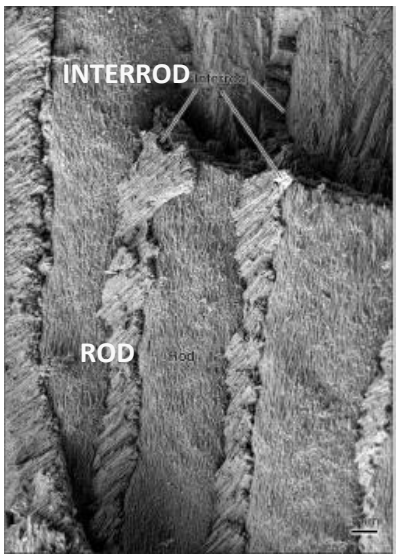


Figure 3.6. *Structure of enamel* (Nanci 2003).

Enamel formation is a very unique process. Traditionally, amelogenesis is described as a four-stage process, including the presecretory, secretory, transition and maturation stages (Fig. 3.7). These stages are associated with a different appearance and function of the ameloblasts concomitant with the production of various enamel components (Bartlett 2013b).

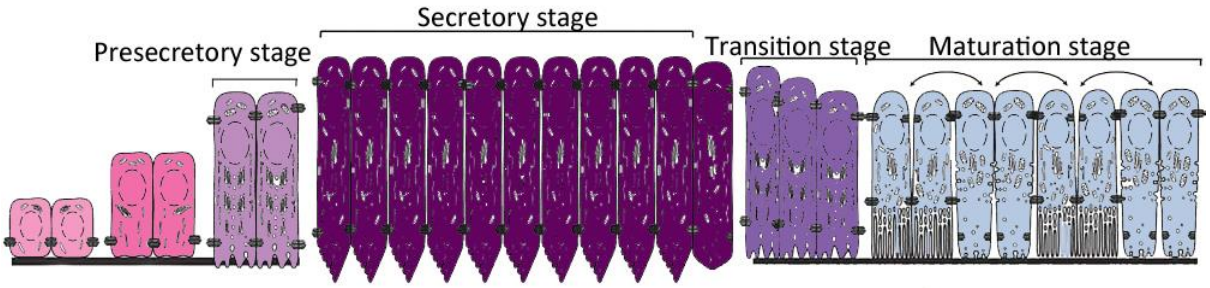


Figure 3.7. *Amelogenesis (adapted from Hu et al., 2007).*

Ameloblast precursors are separated from the forming dentin by a basement membrane. During the presecretory stage, ameloblast precursors differentiate into polarizing ameloblasts that develop a junctional complex at their apical part that assembles an extensive protein synthetic apparatus (Lacruz et al. 2010). The initiation of enamel formation is marked by the degradation of the basement membrane and the initiation of enamel protein deposition. This first layer of enamel has an aprismatic appearance (Hu et al. 2007).

At the secretory stage ameloblasts are highly polarized and show a tall columnar morphology (Fig. 3.5, 3.7). Their nuclei and mitochondria are localized at the outer edge while the endoplasmic reticulum and Golgi complex are located at the opposite secretory edge that forms process called the Tomes' process. At this stage, ameloblasts secrete the specific enamel proteins, which are localized on top and around of the crystallites (Simmer et al. 2012; Hu et al. 2007). Crystallites of rod enamel are formed at the apical most portion of Tomes' process, while crystals of interrod enamel form at their proximal portion. The whole layer of enamel consist of many rods, each of them being a reflection of one ameloblast moving away from the dentin-enamel junction (Ang et al. 2012; Lacruz et al. 2010). To keep high mineral levels within enamel, the organic material should be removed, a process that is realized by the secretion of proteolytic enzyme (Hu et al. 2002). The aim of the transition and maturation stages is to accomplish enamel formation by increasing enamel hardness. At these stages, ameloblasts decrease in height, lose Tomes' processes, and reduce protein synthesis. Ameloblasts acquire ruffle-ended and smooth-ended forms that alternate constantly. Ruffle-ended ameloblasts have endocytotic activity, while smooth-ended ameloblasts provide small proteins and molecules to the forming enamel (Bartlett 2013a; Lacruz et al. 2010). Ameloblasts secrete proteolytic enzyme to remove enamel proteins from the extracellular matrix (Bartlett 2013a). In the empty space created by this mechanism, crystals start growing in width and thickness, occupying thus as much space as possible within the enamel layer. This process promotes the hardening of the enamel layer (Hu et al. 2002; Hu et al. 2007). Between the enamel surface and the ameloblasts an "atypical basal lamina" (BL) is formed, that provides attachment of ameloblasts to the mineralized tooth

surface. Ameloblasts attach to the BL by hemidesmosomes. The term atypical is attributed because this BL does not contain the typical components of basement membrane (BM) such as γ1-chaincontaining laminins, type IV and VII collagens, but laminin-332, Amelotin (Amtn), Odontogenic ameloblast-associated protein (Odam), and Secretory calcium-binding phosphoprotein-proline-glutamine-rich 1 protein (Scpppq1) (Dos Santos Neves et al. 2012; Moffatt et al. 2014; Hormia et al. 2001).

Organic composition of enamel – enamel proteins

The organic matrix of enamel is composed by enamel proteins and enzymes (Table 3.1). Three proteins are the main enamel proteins: Amelogenin (Amg), Ameloblastin (Amb), and Enamelin (Enam) (Bartlett 2013a). Their function in enamel development is to provide support for growing hydroxylapatite crystals during the secretory stage. Amg forms 90% of all extracellular matrix proteins secreted by ameloblasts (Gibson et al. 2001). Chromosomal localization of *Amg* varies between species: while mice have one *Amg* gene, localized on the X chromosome, humans have two genes, which are localized on both X and Y chromosomes (Bartlett 2013a). Amg is not essential for the initial steps of enamel mineralization like other enamel proteins, but is mostly involved in crystallite growth. Various splicing forms of Amg have been reported so far (Sasaki & Shimokawa 1995). Amb is important for maintaining the differentiation state of ameloblasts serving as a cell adhesion molecule. Enam is thought to be a critical component of the mineralization front, which is required for the extension of enamel crystallites. Enam and its cleavage products comprise 5% of the total enamel matrix. The C-terminal part of Enam is secreted at the mineralization front, while cleavage products are localized at the developing enamel layer (Hu et al. 2008).

Proteases that are involved in the degradation of enamel proteins include Mmp20 and Klk4. Mmp20 is an enamel protease expressed during the secretory and transition stages of amelogenesis (Seymen et al. 2015). It was shown that Mmp20 cleaves all enamel proteins. Furthermore, Mmp20 cleaves the Klk4 propeptide, a process that is necessary for obtaining the catalytically active form of Klk4 (Bartlett 2013a). Klk4 is expressed during the transition and maturation stages of amelogenesis (Seymen et al. 2015).

Enamel proteins	Physical characteristics
Amelogenins	Major secretory forms: 25kDa, 23kDa, and 20kDa N-terminal domain is tyrosine rich (tyrosine rich amelogenin polypeptide) One phosphate occurs on serine 16 Central part of the protein is leucine rich (leucine-rich amelogenin polypeptide)
Ameloblastin (also known as amelin or sheathlin)	Native: 62 to 70kDa in deep zones fragmented of 13 to 17kDa in peptides
Enamelin	Native: 140 to 159kDa (pig enamelin) ↑89 and 65kDa ↑32kDa in deep regions phosphorylated at 3 sites, O-glycosylated
Sufated glycoprotein	65kDa in the rat N-glycosylated
Tuftelin	55kDa

Table 3.1*. Enamel proteins (Nanci 2003).*

Genes affecting enamel formation

Mutations in enamel proteins and proteases lead to the development of defective enamel.

Lack of *Amg* results in the reduction of enamel thickness. *Amg* null mice exhibit enamel with crystallites that are reduced in width, thickness, and length (Wright et al. 2011; Gibson et al. 2001). *AMG* mutations in humans affect enamel to various extents, according to the mutated part of the gene (Gibson et al. 2001). Indeed, the phenotype of mutations in the N-terminal part of the gene differs from mutations occurring at the C-terminal part, thus affecting differently enamel density, volume, and organization (Pugach et al. 2010; Lokappa et al. 2015).

In *Amb* mutant mice the enamel appears as a rough thin layer without any prism pattern. Ameloblasts in *Amb* KO mice reach the secretory stage and produce the first layer of enamel but soon after detach from the enamel matrix and lose their polarity (Fukumoto et al. 2004). Furthermore, adult *Amb*-null mice develop tooth-related tumors: ameloblasts dedifferentiate escape from the tooth and form tumors in the soft oral tissues (Wazen et al. 2009; Fukumoto et al. 2004). A similar effect has been observed in humans, where mutations in *AMB* may lead to ameloblastoma, adenomatoid, and squamous odontogenic tumors (Perdigão et al. 2009; Perdigão et al. 2004). When *Amb* is overexpressed in mice, the non-mineralized components are not sufficiently removed from the matrix, thus leading to enamel defects (Smith & Chun 2015).

Mutations in *Enam* in mice lead to the formation of very thin and disorganized enamel that disintegrates quickly after tooth eruption (Masuya et al. 2005; Hu et al. 2008). The enamel does not form the appropriate prism structures and exhibits a rough surface (Wright et al. 2008). Furthermore, ameloblasts of *Enam*-deficient mice undergo premature and extensive apoptosis. The function of Enam is dosage-dependent: heterozygote *Enam*-mice show a milder phenotype than homozygote

mutants. Mutations of *ENAM* in humans are also dosage dependent, varying from localized pitted enamel to very thin enamel (Hu et al. 2014; Ozdemir et al. 2005; Wright et al. 2008). *Enam* overexpression in mice leads to defects in the production of enamel crystals and prism structures (Hu et al. 2014).

In *Mmp20* mutant mice the overall enamel mineral is reduced by 50% and the enamel hardness by 37% when compared to the enamel of wild type animals. Mutations in *Mmp20* affect ameloblasts at the secretory stage. In humans, mutations in *MMP20* are responsible for enamel defects that are similar to those observed in mice (Bartlett 2013a).

Mutations in *Klk4* lead to enamel defects in mice, which appear only at the transition and early maturation stage of amelogenesis (Bartlett 2013a). The deletion of Klk4 has as consequence the non-removal of the enamel proteins from the deposited matrix, thus leading to the development of a soft enamel of normal thickness (Bartlett 2013a). In absence of Klk4, Mmp20 is observed at the maturation stage of ameloblasts, suggesting that Klk4 may hydrolyze Mmp20 (Bartlett 2013a). In humans mutations in *KLK4* lead to the development of enamel which has normal thickness, but the enamel is soft and discolored (Bartlett 2013a; Seymen et al. 2015).

Mutations in other genes involved in enamel development could also lead to enamel defects. For example, Amtn together with Odam protein and Scpppq1 are involved in the formation of the basal lamina, thus allowing the attachment of ameloblasts to the mineralized tooth surface (Moffatt et al. 2014). In mice mutations of *Amtn* lead to the development of hypomineralized enamel that also shows structural defects due to a delay in enamel mineralization (Nakayama et al. 2015). During the maturation stage, ameloblasts are involved in ion transport (e.g., calcium and phosphate) and the control of intra- and extracellular pH (e.g., bicarbonate and hydrogen ion movements) (Lacruz et al. 2012). Mutations in genes involved in these processes have been reported to also affect enamel development (Lacruz et al. 2010).

Manifestation and classification of defective enamel (Amelogenesis Imperfecta)

Amelogenesis Imperfecta (AI) is a clinical term used for hereditary developmental malformations that affect the structure and appearance of enamel (Crawford et al. 2007; Mitsiadis & Luder 2011). In humans, AI is detected both in primary and secondary dentition. The frequency of AI varies from 1:700 to 1:14000 depending on the country studied (Crawford et al. 2007). There are different types of AI classifications, the most common being based on the phenotype and stage of amelogenesis disruption. This classification divides AI in three types: hypoplastic, hypomaturated, and hypocalcified (Fig. 3.8) (Aldred et al. 2003; Crawford et al. 2007). The hypoplastic type of AI is

associated with mutations in enamel proteins that lead to defects in the secretory activity of the ameloblasts. Although the enamel is thinner, its mineralization is not affected. In hypocalcified AI the enamel is of normal thickness but the mineral content is reduced while the protein content is increased. Failure in enamel protein removal during amelogenesis leads to hypomaturated AI, where the enamel is of normal thickness, soft and discolored. This condition is associated with mutations in proteases secreted at the maturation stage of amelogenesis (Crawford et al. 2007).

Figure 3.8. *Types of AI (Crawford et al. 2007; Kim et al. 2006).*

Since AI is a heterogeneous group of disorders resulting from mutations in different genes, in the future gene and mutation-based enamel treatment could be applied in clinics (Alachioti et al. 2014).

3.2 The FAM20 family of protein kinases

The family with sequence similarity 20 (Fam20) comprises three members: *Fam20a*, *Fam20b*, and *Fam20c*. These genes share high homology between human, mouse, and rat. The *Fam20a* gene contains 11 exons, the *Fam20b* contains 7 exons, and the *Fam20c* contains 10 exons. Initially, members of Fam20 family were thought to play roles in regulating the differentiation and function of hematopoietic tissues, but nowadays they are mostly considered to affect the biomineralization processes (Nalbant et al. 2005; Vogel et al. 2012).

	Size (amino acids)	*Molecular mass (Da)*
Fam20a	541	61417
Fam20b	409	46432
Fam20c	584	66234

Table 3.2. Members of Fam20 family.

Structure of Fam20 kinases

Sequence analysis of Fam20 family members revealed several signature amino acid motifs that define protein kinases (Fig 3.9) (Vincent S Tagliabracci, Xiao, et al. 2013). Functional value of these motifs was studied using the *Fam20c* mutants. Glycine-rich loop covers the Adenosine triphosphate (ATP) -binding pocket. Mutation of the threonine residue to methionine within the loop in the *Fam20c* gene causes ectopic calcifications, and dental abnormalities (Rafaelsen et al. 2013). The conserved lysine residue in β3 and a glutamate residue in αC form an ion pair. The function of the lysine residue is the orientation of α- and β-phosphates of ATP in the active site of protein kinase, while the glutamate residue stabilizes the conformation of the lysine residue. Mutations of these residues in *Fam20c* impairs ATP binding and thus protein kinase activity (Vincent S Tagliabracci, Xiao, et al. 2013). Mutation in the catalytic loop of Fam20c completely abolishes its activity, demonstrating its crucial role in the kinase functioning (Vincent S Tagliabracci, Xiao, et al. 2013). The divalent cation that is necessary for the catalysis of the kinase is coordinated by the aspartate residue in the DFG motif. Mutation of this residue in *Fam20c* abolishes its activity (Tagliabracci, Engel, Wen, Wiley, C. a Worby, et al. 2012). Apart from the protein kinase domains, all members have a signal peptide sequence, predicting their roles as secreted protein kinases.

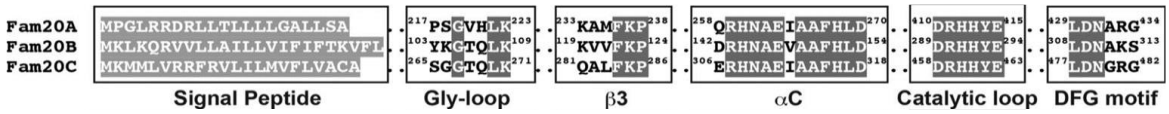


Figure 3.9. Sequence alignment of the Fam20 family of kinases (Vincent S Tagliabracci, Xiao, et al. 2013). Abbreviations : Gly-loop – glycine loop, β3 – β-sheet 3, αC – αC helix, DFG – highly conserved motif Asp-Phe-Gly.

Expression of Fam20 members

Using cDNA derived from various human tissues, it was shown that *FAM20A* is expressed at high levels in lung and liver, at intermediate levels in the thymus and ovary, and at very low levels in some other organs (Fig. 3.10) (Nalbant et al. 2005). In mice *Fam20a* expression was only detected in teeth and in parathyroid gland. In tooth *Fam20a* was strongly expressed in secretion-stage ameloblasts and odontoblasts, and less in the mature ameloblasts of the postsecretion stage (Vogel et al. 2012).

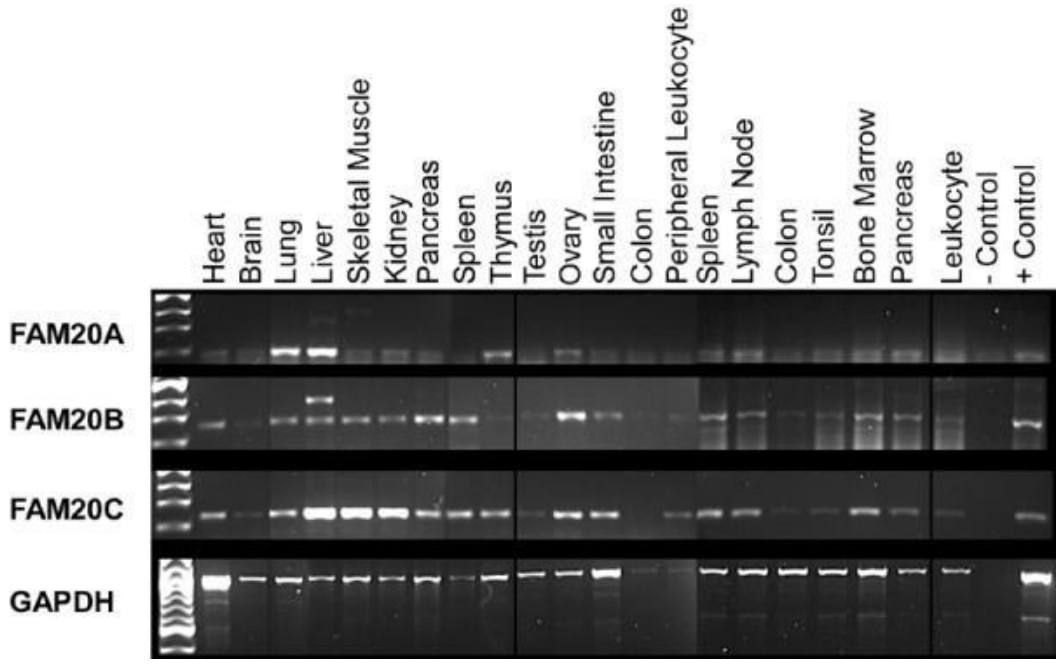


Figure 3.10. *RT-PCR analysis of human FAM20 mRNA levels in human tissues* (Nalbant et al. 2005)

In humans *FAM20B* is expressed in the large variety of tissues, among which the highest level of expression is detected in ovary and pancreas (Nalbant et al. 2005).

In humans *FAM20C* is also expressed in the large variety of tissues including heart, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, ovary, small intestine, and bone marrow (Nalbant et al. 2005). In mice *Fam20c* expression was also investigated during the development of mouse bone and teeth. During bone development *Fam20c* gene is expressed in chondrocytes and osteoblasts of the long bone. The Fam20c protein in its turn is expressed in extracellular matrix of the bone and in the cytoplasm of chondrocytes, osteoblasts, and osteocytes. During tooth development *Fam20c* gene is expressed in ameloblasts, odontoblasts, cementoblasts, and periodontal ligament fibroblasts, same as Fam20c protein. Moreover, Fam20c protein was detected in mineralized parts of the tooth such as enamel, dentin, and alveolar bone (Wang et al. 2010).

Mechanism of action of Fam20 members

Although the Fam20 family contains three members, the only information we have comprises Fam20c. Fam20c is a protein kinase which phosphorylates many secreted proteins, including enamel proteins. The high degree of sequence homology between *Fam20a* and *Fam20c* brought to the assumption that Fam20a has a similar mechanism of action as Fam20c. However, some results have shown that Fam20a cannot phosphorylate the proteins. A step-by-step investigation has showed that Fam20a can bind ATP but is not able to hydrolyze it. It was shown that Fam20a potentiates kinase

activity of Fam20c and promotes phosphorylation of enamel proteins. Fam20a and Fam20c form a functional heterotetrameric complex composed of two Fam20a and two Fam20c subunits. In this complex the Fam20c is catalytically active while the Fam20a serves as an activator of the Fam20c kinase activity. This complex catalyzes substrate phosphorylation much more efficiently than the Fam20c alone (Cui et al. 2015).

The detailed mechanism of action of these secreted protein kinases is still under investigation. A mechanism of action for Fam20c has been proposed: phosphorylation occurs in the lumen of Golgi or extracellularly, since both the kinase and its targets are secreted proteins (Fig. 3.11) (Vincent S Tagliabracci, Pinna, et al. 2013). It has already been shown that Fam20c can phosphorylate intracellularly, but this can also happen extracellularly (Vincent S Tagliabracci, Pinna, et al. 2013; Tagliabracci et al. 2015a; Tagliabracci, Engel, Wen, Wiley, C. A. Worby, et al. 2012).

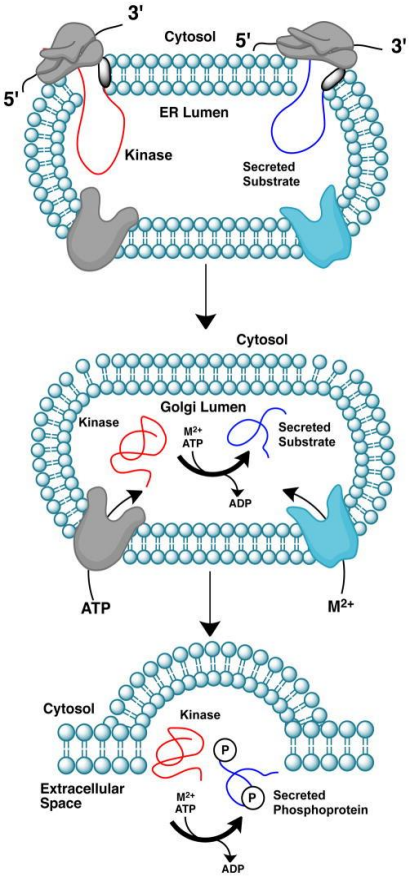


Figure 3.11. *Family of secretory calcium binding phosphoproteins (SCPPs)* (Vincent S Tagliabracci, Pinna, et al.

Targets of Fam20a and Fam20c

More than 100 target proteins have been identified for Fam20c protein kinase. These targets include proteins involved in biomineralization processes, lipid homeostasis, wound healing, cell migration, and cell adhesion (Tagliabracci et al. 2015a). Fam20c phosphorylates proteins on S-x-e

motifs (Ser-x-Glu/phosphoSer), including casein, FGF23, and members of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family (Fig. 3.12) (Tagliabracci, Engel, Wen, Wiley, C. A. Worby, et al. 2012)(Tagliabracci et al. 2015a; Cui et al. 2015; Tagliabracci, Engel, Wen, Wiley, C. A. Worby, et al. 2012).

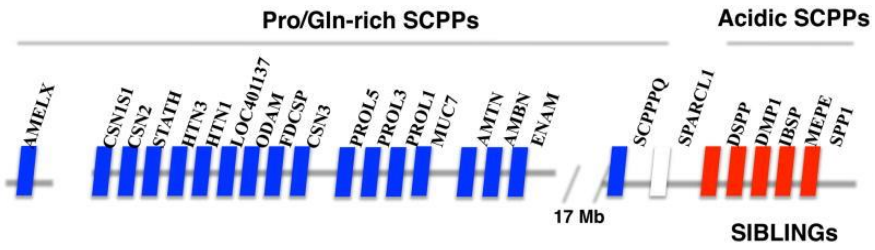


Figure 3.12. *Targets of Fam20c* (Vincent S Tagliabracci, Pinna, et al. 2013).

Defects caused by mutations in Fam20 members

Fam20a

Dental defects associated with mutations in *FAM20A* started to be described since 2008 (Table 3.3). In these studies, *FAM20A* patients exhibit hypoplastic AI together with gingival hyperplasia, teeth with intrapulpal calcifications, and delayed or failed tooth eruption. Some of these patients exhibited teeth smaller than normal size. Recently, a study combining analysis of 16 families demonstrated that all patients exhibited nephrocalcinosis (Jaureguiberry et al. 2012). However, previous and recent studies have indicated that nephrocalcinosis is not always associated with dental defects caused by mutations in the *FAM20A* gene (S. K. Wang et al. 2014; Cherkaoui Jaouad et al. 2015; Kantaputra et al. 2014).

Table 3.3.*Summary of all published cases of Fam20a mutations around the world with described phenotype and mutation details. AR – autosomal recessive inheritance.*

Country	Oral cavity defect	Nephrocalcinosis	Family #	Gender		Age	Gene	Reference
Moroccco	Hypoplastic AI in primary and permanent dentition Little or no enamel on all teeth Crowns are short Teeth colour yellow-brown Generalized severe gingival hyperplasia Supra-incisive diastema	no		1 Female	AR	11	Exon 1	(Cherkaoui Jaouad et al. 2015)
Turkey, Korea	AI Severe generalized gingival hyperplasia Calcified materials in the pulp chambers Prolonged retention of the deciduous teeth Delayed or failed eruption of permanent teeth Dilacerations of the root Agenesis of some teeth	?	1	1 Female	AR		Exon 1	(Cho et al. 2012)
			4	1 Female	AR		Intron 2 Exon 6	
	AI Mild localized gingival hyperplasia Calcified materials in the pulp chambers Prolonged retention of the deciduous teeth Delayed or failed eruption of permanent teeth Dilacerations of the root Agenesis of some teeth	?	2	1 Female 3 Male	AR		Intron 5	
			3	2 Male	AR		Exon 8	
UK	Generalized hypoplastic AI (4/4) Gingival fibromatosis (4/4) Pericoronal radiolucencies, unerupted teeth (4/4) Intrapulpal calcifications (4/4) Retention of deciduous teeth (3/4) Dental agenesis (2/4) Root dilacerations (1/4) Microdontia (1/4)	No		Female	AR	19	Exon 2	(Martelli-Júnior et al. 2008; O'Sullivan et al. 2011)
				Female		13		
				Female		13		

				Male		18		
Caribbean	Hypoplastic AI Gingival hyperplasia Intrapulpal calcifications Failed or delayed eruption of some teeth Mixed dentition	?	1	2 Female 1 Male			Exon 7	(S.-K. Wang, Aref, et al. 2013)
Jordan	Hypoplastic AI Gingival enlargement Failed or delayed eruption Pulp calcifications Apparent crown resorption	Yes	2	1 Male			Intron 4	
Iran	Lack of enamel Delayed tooth eruption Intrapulpal calcifications Pericoronal radiolucencies Apparent crown resorption	?	3	1 Female 1 Male			Exon 2 Exon 11	
Irish/ Norwegian	Hypoplastic AI Normal size gingiva Secondary teeth small and misshapen Failed or delayed in eruption Pulp stones	No	1	1 Female		13	Intron 1 Exon 8	(S. K. Wang et al. 2014)
Mexican	Hypoplastic AI Mild or absent gingival hyperplasia Pulp stones Failed or delayed eruption	?	2	1 Female 1 Male			Boarder of Exon 10 and Intron 10	
Pakistani	Gingival hyperplasia Delayed tooth eruption Failure of tooth development	No	1	2 Female 10 Male			Duplication in Exon 1	(Cabral et al. 2013)
	Hypoplastic AI Gingival hyperplasia Impaired tooth eruption	Yes	1	1 Male		21	Exon 6	(Jaureguiberry et al. 2012; de la Dure-Molla et al. 2014)
			2	1 Female 1 Male		27 31	Intron 2 Exon 6	
			3	1 Female 1 Male		25 23	Intron 4 Exon 10	
			4	1 Female 1 Male		64 59	Exon 11	

			5	1 Female		12	Exon 2	
			6	1 Male		20	Exon 1	
			7	1 Female 1 Male		16 22	Exon 11	
			8	1 Male		20	Exon 11	
			9	1 Male		13	Exon 2	
			10	1 Female		29	Exon 5 Exon 9	
			11	1 Female 1 Male		19 20	Exon 1 Exon 5	
			12	1 Female		18	Exon 11	
			13	1 Female 1 Male		14 16	Exon 4 Exon 5	
			14	1 Female		21	Intron 5	
			15	1 Female 2 Male		37 24, 31	Exon 6	
			16	2 Female		17, 18	Exon 1 Exon 3	
Turkish	Hypoplastic AI Gingival fibromatosis Malposition of permanent teeth Prolonged retention of primary teeth Failed or delayed eruption Very large dental follicles	No		1 Male		17	Exon 1	(Kantaputra et al. 2014)
Turkish	Hypoplastic AI Gingival fibromatosis Malposition of permanent teeth Prolonged retention of primary teeth Failed or delayed eruption Heterotopic calcification in dental pulp	Yes		1 Female			Exon 11	

Fam20b (also known as xylose transferase I)

Fam20b phosphorylates the xylose residue in the glycosaminoglycan (GA)-protein linkage region within proteoglycan (PG), and thus regulates the number of GA chains (Koike et al. 2009). Fam20b is very different from the Fam20a and Fam20c proteins. The role of Fam20b in skeletal formation has been investigated for the first time in zebrafish (*Danio rerio*). Fam20b was shown to have an important function in PG synthesis. Mutations of Fam20b caused severe skeletal defects due to the reduction of PG synthesis in the cartilage (Eames et al. 2011). Fam20b is also involved in liver regeneration and blood vessel remodeling by enhancing GA biosynthesis (Nadanaka & Kitagawa 2014; Nadanaka et al. 2013). The attempt to study the role of Fam20b in mice failed due to the embryonic lethality of the Fam20b mutant mice (Vogel et al. 2012). At E12.5 Fam20b KO embryos are small, exhibit multisystem organ hypoplasia, and considerable delay in the development of the eye, lung, gastrointestinal tract, and liver (Vogel et al. 2012).

Fam20c (also known as Dmp4)

Raine syndrome is a rare recessive autosomal disorder characterized by osteosclerotic bone dysplasia (Faundes et al. 2014; Simpson et al. 2009). Apart from generalized osteosclerosis patients with Raine syndrome also exhibit brain abnormalities, cleft palate, hyperplastic gums, and a characteristic facial phenotype (Faundes et al. 2014; Mddica 1988). Raine syndrome has been associated only with mutations in *FAM20C*. Mutations could be divided into two groups: those that affect FAM20C secretion and those that affect the kinase activity (Faundes et al. 2014). Detailed investigations showed that mutations in *FAM20C* led to reduced kinase activity and secretion (Takeyari et al. 2014).

The role of Fam20c in tooth formation was studied using Sox2-Cre mediated *Fam20c* conditional knock-out mice. All mineralized compartments of the teeth were affected: enamel, dentin, cementum, and the surrounding alveolar bone. The enamel was thin, irregular, loosely attached to the dentin, and detached from the ameloblasts. Ameloblasts depolarized and disorganized completely. Odontoblasts also lost their polarity and produced thin and porous layers of dentin. *Amb* and *Amg* expresseion was strongly downregulated in the ameloblasts of mutants. Together with the defects in mineralized tissues, other dental defects were observed: short incisors, undeveloped roots, malformed cusps, and enlarged pulp chambers (Xiaofang Wang, Wang, Lu, et al. 2012). The role of *Fam20c* in enamel development was investigated using a mouse model with a deletion of *Fam20c* in epithelial cells. It was reached by the crossing of Fam20Cfl/fl mice with keratin 14-Cre (K14-Cre) transgenic mice. Mouse mutants exhibit severe enamel defects showing very thin and poorly calcified enamel with no clear rod structure. In situ hybridization (ISH) and Real-time polymerase chain reaction (PCR) showed downregulation of the main enamel proteins *Amg* and *Amb*

(X. Wang et al. 2013). To check the role of *Fam20c* in the formation of dentin and alveolar bone transgenic mice were used, in which *Fam20c* expression was abolished from the neural-crest-derived craniofacial tissues that form dentin and alveolar bone by use of transgenic mice Wntl-Cre;Fam20C$^{fl/fl}$. Dentin and bone formation were highly affected in these transgenic mice. Dentin was much thinner and less mineralized than normal dentin. The alveolar bone also appeared poorly mineralized. Significant down-regulation of dentin markers such as *Dmp1* and *Dspp* was also found (Wang et al. 2015). To investigate the role of *Fam20c* in periodontal tissues mutant mice were generated, in which type I collagen was inactivated. Severe bone defects, disorganization of collagen fibers of the PDL, inflammation in PDL, and the formation of deep periodontal pockets were observed (Liu et al. 2014).

4. Aims of the project

1. To describe *Fam20a* expression in developing teeth.

The starting point for the study of a new gene involved in tooth defects is to describe its expression in the developing tooth starting from the early embryonic stages followed by tooth maturation in detail.

2. To study the role of *Fam20a* in enamel formation.

To investigate the role of *Fam20a* deletion on enamel development using *Fam20a* mutant mice.

3. To investigate the regulation of *Fam20a* expression.

To performe recombination experiments in order to understand the role of *Fam20a* expression in different tooth compartments on enamel development.

4. To rescue the Fam20a phenotype.

We produce the FAM20A recombinant protein and then by addition of this protein to the developing *Fam20a* mutant mouse teeth we rescue the enamel defect.

5. Materials and methods

5.1 Mice

Animal housing and experimentation were performed according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich.

Wild type C57/BL6J mice were used at different embryonic and postnatal stages. *Fam20a* mutant mice were generated and provided by Prof. Aris Economides, Regeneron Pharmaceuticals, Inc. In these mice the first exon and a part of the first intron were replaced by a LacZ cassette.

The day a vaginal plug is detected was counted as embryonic day 0 (E0), and the day of birth was considered as postnatal day 0 (P0). The stages of embryonic development were confirmed according to morphological criteria.

Mouse genotyping was performed via PCR using the following primers:

Forward: TGTCAACACTGTGAGCTTCC

Reverse 1 (WT): CCTGAAAAGTTGTGCGAGGC

Reverse 2 (KO): GTGTAGATGGGCGCATCGTA

5.2 Tissue collection and culture

Dissection

Embryonic tissues for various analyses were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA). The time of fixation depends on the tissue sample and the procedure (1h – 1 day). Pups and adult mice were perfused intracardially with 4% PFA; the head was then post-fixed in 4% PFA for 4 to 8 weeks.

Decalcification

Samples from adult mice were decalcified in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated, embedded in paraffin and sectioned at 5-10 µm. Decalcification was not performed on embryonic tissues.

Semi-solid plates for tooth germ cultures

Semi-solid plates (d=35mm) were prepared on the hot plate (T=64^{o}C). DMEM + 20% FBS + 1% P/S + 1% L-Glutamine + 0,18mg/ml Ascorbic Acid was mixed with 0.2ml of agarose 0,05gr/ml. Plates were then let to solidify at room temperature. Plates were changed every two days.

5.3 Stainings

Haematoxylin/eosin staining

Haematoxylin/eosin staining was performed on paraffin and cryo-embedded sections. Rehydrated sections were incubated for 1'30'' in filtered haematoxylin, washed with tap and bi-distilled H_2O, incubated for 1' in filtered eosin, dehydrated and mounted with Eukitt (Fluka, Ref 03989).

Masson's trichrome staining

Masson's trichrome staining was performed on paraffin sections. Sections were rehydrated, incubated for 1'30'' in filtered haematoxylin, washed with tap and bi-distilled H_2O, incubated with aniline red Ponceau for 10', washed with tap water, dipped in 1% glacial acetic acid, incubated with Phosphomolybdic acid-Orange for 5', dipped in 1% glacial acetic acid, incubated for 1' in light green, dipped in 1% glacial acetic acid, incubated for 30'' in light green, dipped in 1% glacial acetic acid, rehydrated and mounted with Eukitt.

Anti β-galactosidase staining

Samples were fixed with 2%PFA + 0,02% glutaraldehyde, washed with PBS, incubated in staining solution (1mg/ml X-Gal, PBS, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 5mM EGTA, 2mM $MgCl_2$, 0,02% NP-40 in water) at 30^{o}C overnight or untill the staining is visible. Samples were then postfixed in 4%PFA and embedded in paraffin or OCT.

5.4 In situ hybridisation

In situ probe production

Fam20a probe sequence:

CAAGCTCGCCCTGAGTCGCCCCTGCTTTGTCAACTCTGCGTTCCCTCCGCTCCTGCCAGCCCGGGGCACAGG
AGTCCCTGCCGCCTGCAGGAGGAGCCGCTGGATCTGTCTCAGCAGCTGCGGAGAGACAGCATCCTTGGAAGA

GACGCCCCGGGCTACGCAGTCTCTCCAGCCTCCTCCTAGGGGGCGAGAGCTCCTTTGCGCTCCCTCCAGTGATC
GCCCTCTACACGGCCACCAGAGCCAGCACTCCTCCACGGGACCTCCCCACCTGAGCGGTCCCTGGCAGAGGGG
GTCACAGGGCAAAGAGACTCAAATAACAACCGGGTAGCCAGGGAACCCCAACCCTGCCTCTCTACTGGGCAA
GAAAAGACGACGCTGTCAACACTGTGAGCTTCCTGCACGCGGTCCCCAAGTTCAGGGAAGACACCCTTCCAGG
AAGAGCTAATCCCCTGTGTGAGCATTCCAGCCTCCTCCGGACTCCAGGAGCTTCTTCCGTCTGCACCCCAAGTG
TTTCCCGGGTGTCCGCTTGGGCCATGCCCGGGCTGCGCAGGGACCGCCTGCTGGCCCTGCTGCTCCTGGGCGC
GCTCTTCTCCGCCGACCTCTACTTCCACCTCTGGCCGCAAGTGCAGCGCCAGCTCCGGCCGGGCGAGCGCCCCG
CCGCCTGCCCGTGCTCTGGCCGCGCCCCCTCCGCGTCTCTACACTCAGCCGCAGCCTCCCGAGACCTTGGCACA
GCCTCGCACPCR

The selected region was amplified by standard PCR from cDNA (primers A001 and A002), and was cloned into the plasmid pGEM-T Easy (Promega, A1360). Competent bacteria were transformed using the heatshock technique, and pDNA was extracted from the agarose gel using a MiniPrep Kit (Invitrogen, Ref K210010). For the generation of the sense and antisense probes BstXI (Promega, R6471) and ApaI (Promega, R6361) restriction enzymes were used, respectively. The restriction enzyme digestion was set up using standard Promega protocol with suitable restriction enzyme buffer. After linearisation Digoxigenin-UTP labeling was performed using a DIG labeling KIT (SP6/T7) (Roche, 11175025910).

Primers:

A001: CAAGCTCGCCCTGAGTCGCC

A002: GTGCGAGGCTGTGCCAAGGT

In situ hybridisation

In situ hybridisation was performed on paraffin sections or cryosections as previously described (Mitsiadis et al. 1995). For paraffin sections pre-treatment with proteinase K, HCL, and 0.1M triethanolamine-HCl was performed. The labelled probe was diluted in Hybrisol (0,3-1µg/ml in 1ml). The probe was denaturated in Hybrisol for 5-10' at 70^{o}C. In situ hybridisation was performed via incubation with the probe at 63°C overnight. After intense washing in washing solution (1.3x SSC + 50% formamide + 0,1% Tween-20), the slides were incubated in blocking solution (MABT - 100mM maleic acid, 150mM NaCl, 0,1% Tween 20,- at pH=7,5) + 20% Normal Goat Serum). After that the slides were then left overnight at 4°C with anti-DIG antibody (1 in 1000) in the blocking solution, followed by washing with MABT on the next day. The colour reaction was developed using Nitro Blue Tetrazolium (NBT, Sigma N-6876) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, Sigma B-8503) in

staining solution (2% NaCl, 5% $MgCl_2$, 10% Tris-HCl pH 9.5, 1% Tween-20, and 0,5% Levamisole). When the staining intensity reached the required level the slides were washed with PBS + 0.1% Tween-20, fixed in 4%PFA, and mounted.

5.5 Immunohistochemistry

Immunohistochemistry was performed on 5-10µm thick paraffin sections or cryosections. Cryosections were fixed with cold acetone onto the slides; paraffin sections were rehydrated before staining. Endogenous peroxidases were quenched by incubating sections in 3-6% H_2O_2 in ice-cold methanol for 30'. For specific stainings, heat-induced antigen retrieval in 10mM trisodium citrate buffer, pH 6.0, was performed. For the staining procedure, the Vectastain ABC Kit was used (Vector Laboratories,). As chromogenic substrate, SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma-Aldrich, D4293) were used. Omission of the primary antibody served as a negative control. Stained sections were counterstained with toluidine blue or haematoxylin (both diluted 1:10 in distilled H_2O) and mounted with Glycergel (C0563, Dako, Dako North America Inc.) or Eukitt® (Fluka, Sigma-Aldrich Chemie, 03989).

5.6 Microscopy

Pictures were taken using the Leica DFC420C camera and the Leica Application Suite (LAS) software.

5.7 Micro-computed tomography (µCT)

The µCT scans were performed using a commercially available µCT unit (Specimen µCT 40, Scanco Medical, Brüttisellen, Switzerland) with all imaging parameters kept identical during all examinations (tube voltage, 70 kV, tube current 114 µA; isotropic resolution, 18 µm). The original images were converted into the RAW-format using the proprietary software of the µCT device and imported in the 3D reconstruction program VGStudio Max (Volume Graphics, Heidelberg, Germany). All the analysed samples were segmented manually using wild type (wt) teeth as reference for the grey level values corresponding to the single mineralized tissues (enamel, dentin, bone).

5.8 Recombination experiment

Mouse molars of *Fam20a*$^{-/-}$ and wt E14.5 mouse embryos were dissected as described above. Using digestion buffer (20%Dispase + 0,2% Dnase I in HBSS) the epithelium was separated from the

mesenchyme and recombined. Four types of recombinants of epithelium/mesenchyme were prepared: wt/wt, wt/mutant, mutant/wt and mutant/mutant. Recombinants were cultured overnight at 37^{o}C and transferred under the kidney capsule of immune-compromised mice (Rag1$^{-/-}$ (described later) on the next day. After 1 month the kidneys were analysed by μCT, embedded in paraffin, sectioned and stained.

5.9 Kidney capsule transplantation

Adult mice (2-4 months old) were anesthetised with isoflurane inhalation, the back skin and peritoneum opened with microsurgical scissors, and the kidney exposed. The membrane covering the kidney (capsule) was gently opened to create an access point. The samples were transferred underneath the membrane. The kidney was then placed back in its normal position and the peritoneum sutured with absorbable suture. The skin was stitched and the mice were left to recover on a heating pad. The post-surgery treatment was performed with paracetamol (Dafalgan Kinder-Sirup, 7 ml in 60 ml of drinking water); the stitches were removed after 10 days. The experiment was terminated after 4 weeks. The mice were euthanized and the kidney carefully dissected.

5.10 Phenotype rescue experiment

Molars and incisors from *Fam20a*$^{-/-}$ E16.5 mouse embryos were dissected as described before. They were kept for 1 week on semi-solid plates. Twice a day they received 10μl of PEM medium (Gibco, 12661-015) + recombinant *FAM20A* (1 mg/ml). After that samples were placed surgically under the kidney capsule of Rag-/- mice (see above) and left for 3 days, followed by μCT analysis, embedment in paraffin, sectioning and histological analysis.

6. Results

Expression of Fam20a gene during orofacial development

The expression of *Fam20a* in developing teeth and orofacial tissues is nearly unknown. To investigate that, we performed *in situ* hybridization (ISH). ISH allows visualizing the pattern of expression of a studied gene. First, we designed and produced an antisense *Fam20a* RNA probe. During the development of the head *Fam20a* is expressed in various orofacial tissues, including the developing eye, nasal cavities, whiskers, bone, brain, and skin (Fig. 6.1).

To clarify the role of *Fam20a* in tooth development, we analyzed its expression at different

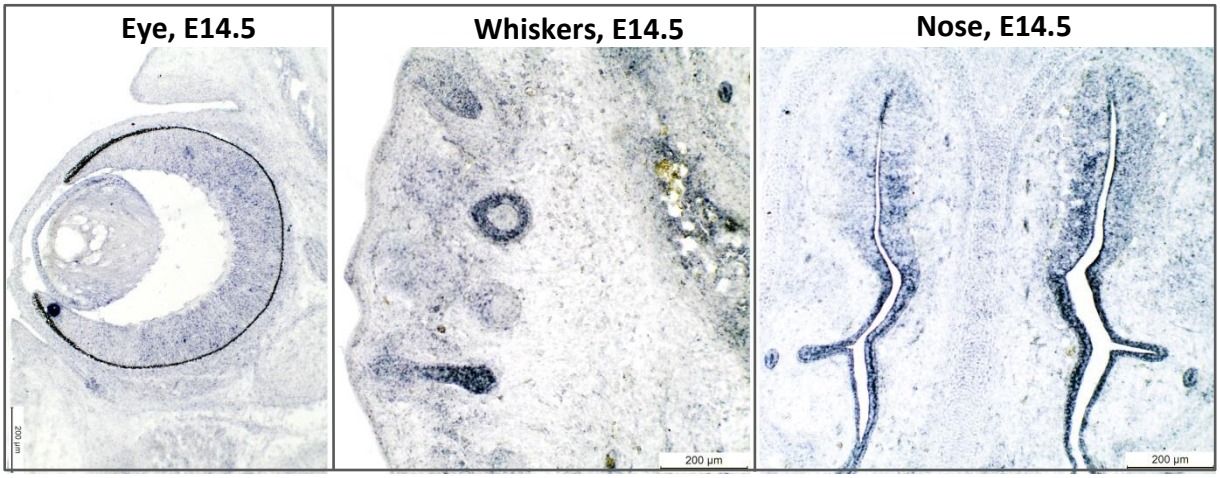


Figure 6.1. *In situ* hybridization showing the expression of *Fam20a* gene in orofacial complex during embryonic development.

embryonic and postnatal stages by ISH. At E14.5 *Fam20a* expression is mostly detected in epithelial tissues of the tooth and also in the oral epithelium (Fig. 6.2). In the tooth epithelium, expression is mostly localized in cells that are on the border of epithelium and mesenchyme. Moreover, strong *Fam20a* expression was observed in forming alveolar bones that will surround the mature tooth at later developmental stages. In mesenchymal tissues of the tooth expression of *Fam20a* is almost missing, despite some point expression.

At E15.5, the *Fam20a* expression in the epithelium is stronger and widely distributed among epithelial cells (Fig. 6.3). Additionally, mesenchymal cells of the tooth start expressing *Fam20a*. In mesenchymal cells Fam20a expression is mostly detected in cells adjacent to the tooth epithelium.

Expression of *Fam20a* in the tooth at E17.5 is still detected in epithelium and adjacent mesenchyme (Fig. 6.4). Differently from previous stages, at E17.5 the highest level of *Fam20a* expression is detected in CL areas, which consist of less differentiated cells. Moreover, *Fam20a* continues to be expressed in the developing alveolar bone that surrounds the tooth.

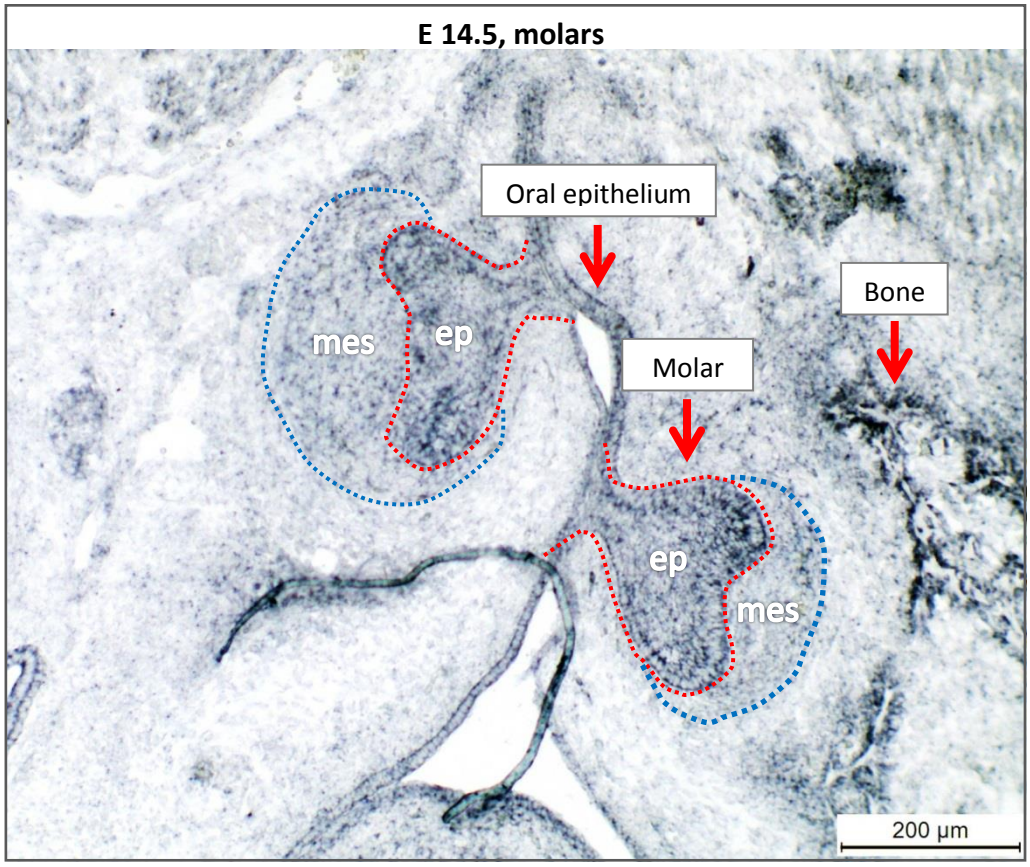


Figure 6.2. *In situ* hybridization showing expression of *Fam20a* gene in mouse molars at E14.5. Abbreviations: ep – epithelium, mes – mesenchyme. Red dots mark epithelium of the tooth, blue dots mark mesenchyme.

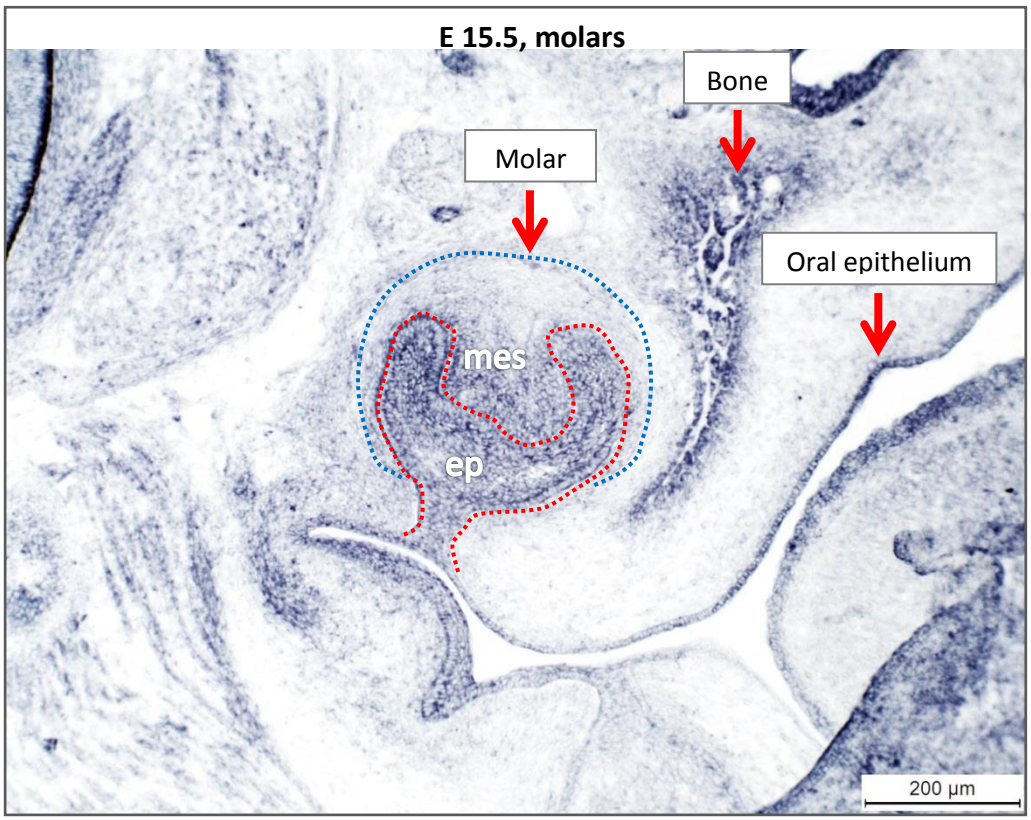


Figure 6.3. *In situ* hybridization showing expression of *Fam20a* gene in mouse molars at E15.5. Abbreviations: ep – epithelium, mes – mesenchyme. Red dots mark epithelium of the tooth, blue dots mark mesenchyme.

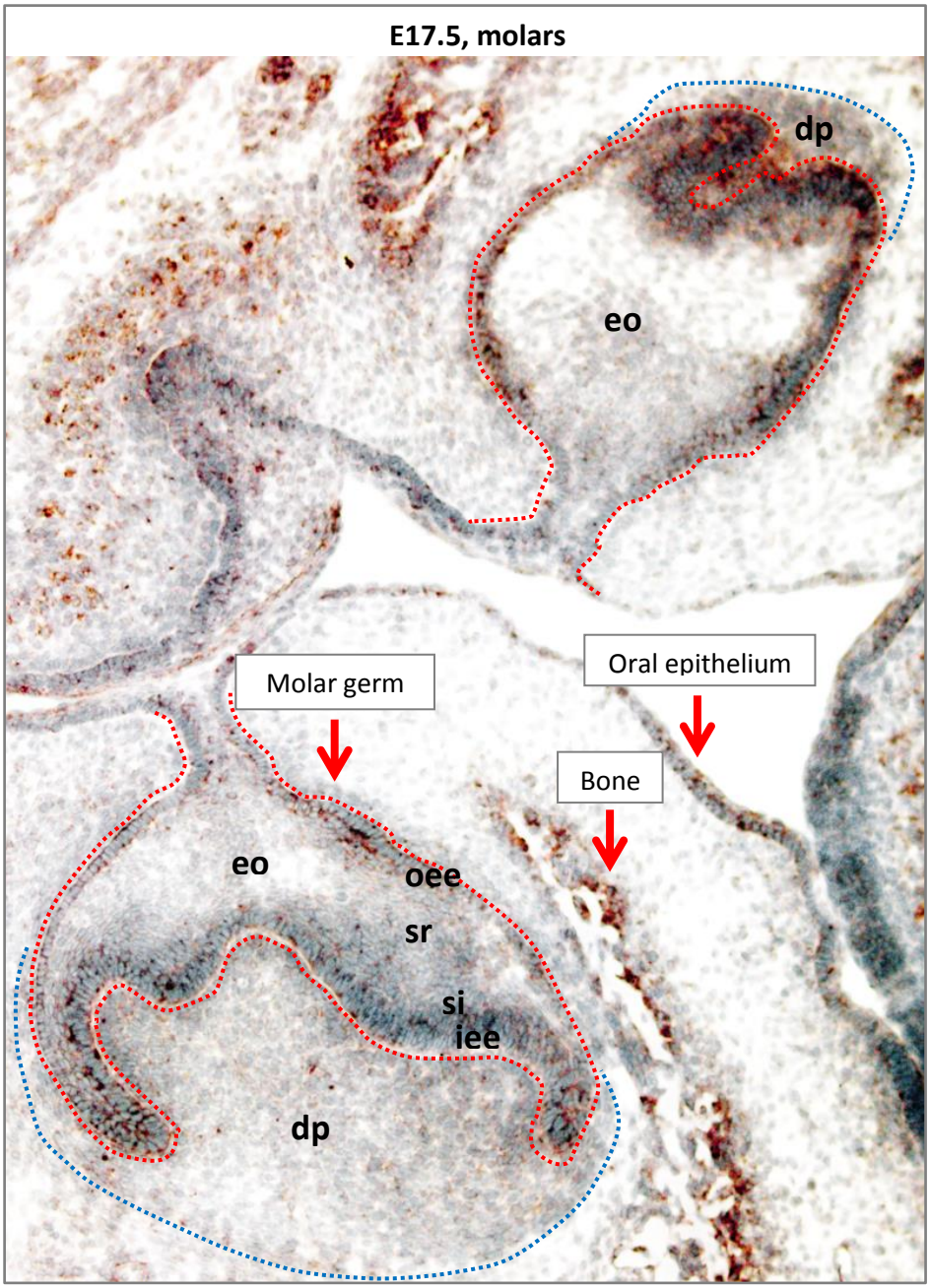


Figure 6.4. *In situ* hybridization showing the expression of *Fam20a* gene in mouse molars at E17.5. Abbreviations: dp – dental papilla, eo – enamel organ, iee – inner enamel epithelium, oee – outer enamel epithelium, si – stratum intermedium, sr - stellate reticulum .

At postnatal stage 0 (P0) the expression of *Fam20a* is specific to certain parts of the tooth (Fig. 6.5). It is very strong in preodontoblasts and weaker in preameloblasts. Furthermore, *Fam20a* expression is observed in the dental follicle, which surrounds the tooth.

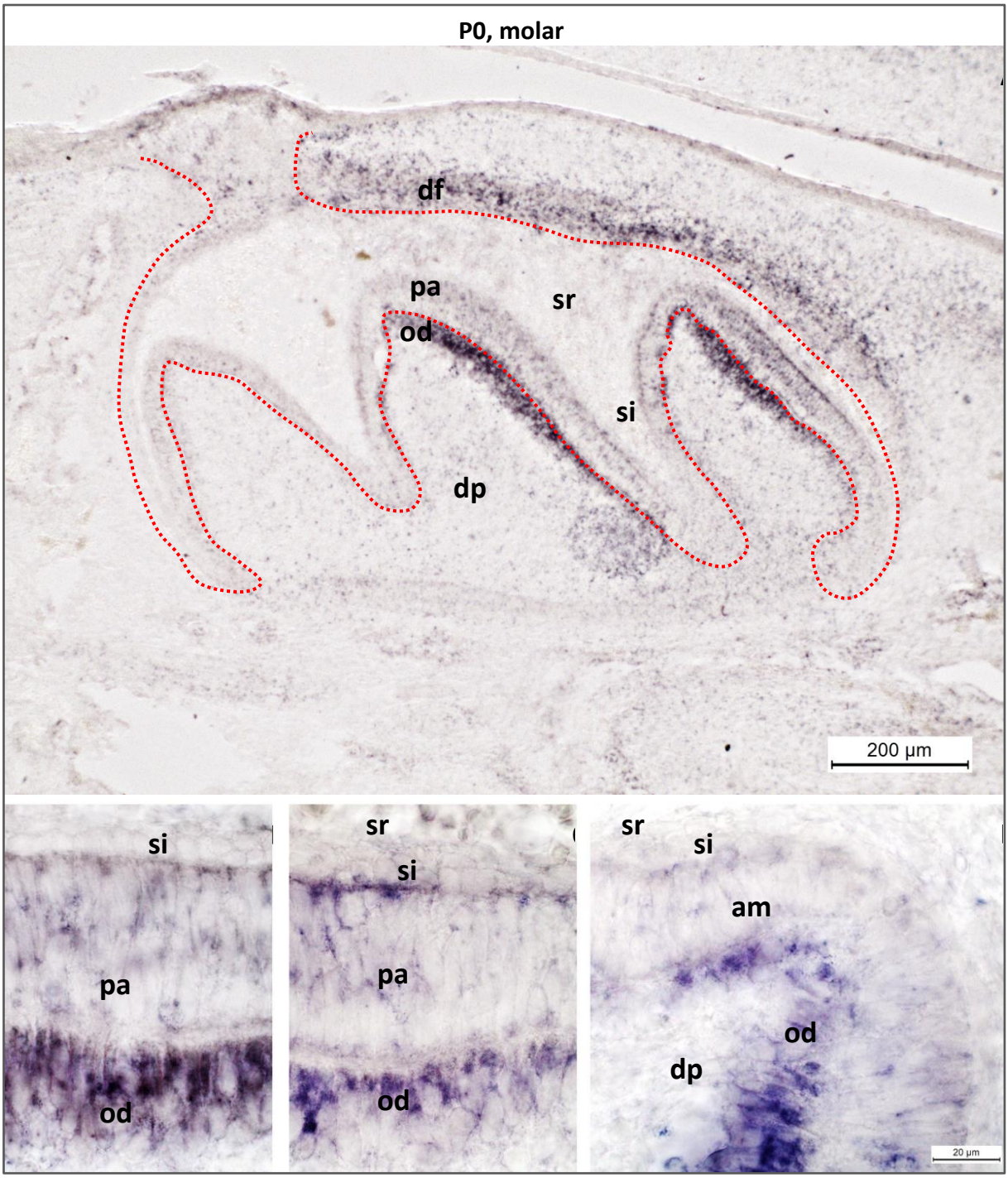
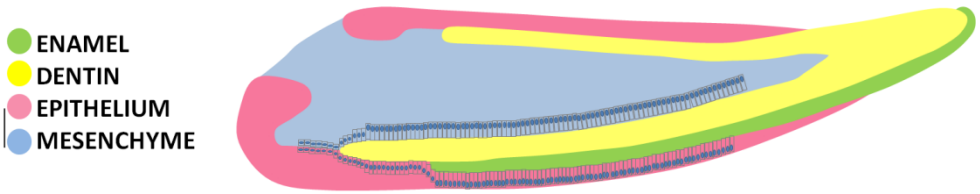


Figure 6.5. *In situ* hybridization showing the expression of *Fam20a* gene in mouse molars at P0. Abbreviations: am – ameloblasts, df – dental follicle, dp – dental papilla, eo – enamel organ, iee – inner enamel epithelium, od – odontoblasts, oee – outer enamel epithelium, pa – preameloblasts, si – stratum intermedium, sr - stellate reticulum .



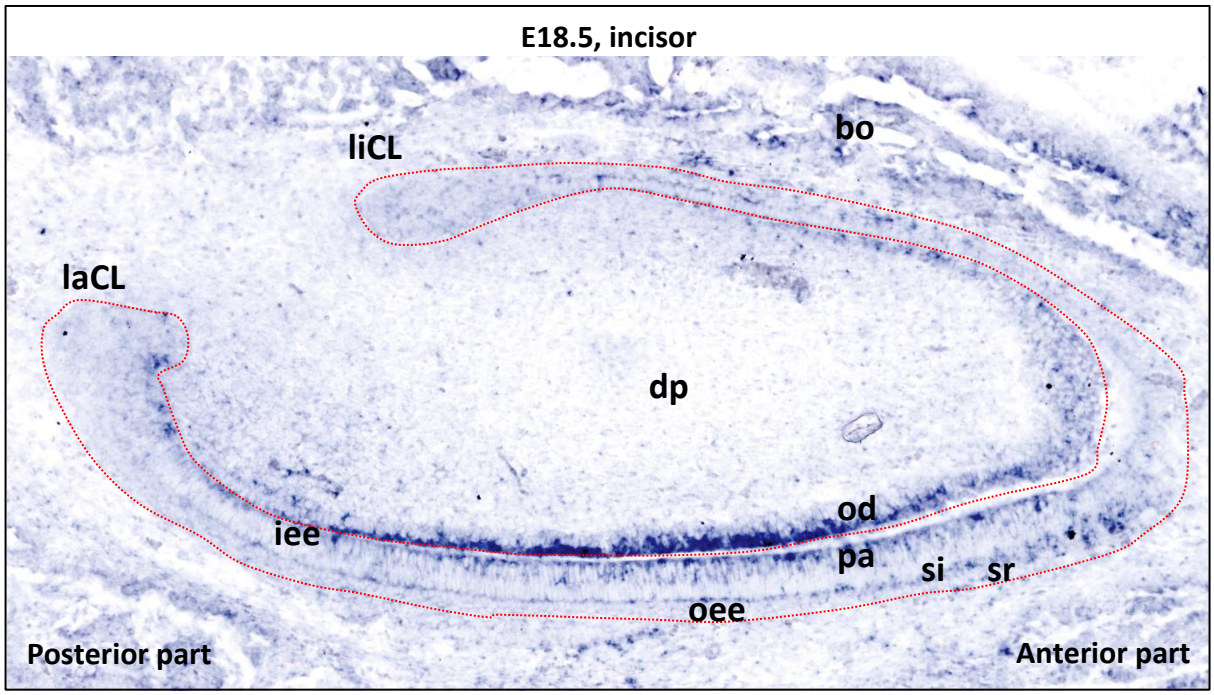


Figure 6.6. *In situ* hybridization showing the expression of *Fam20a* gene in a mouse incisor at E18.5. Abbreviations: dp – dental pulp, iee – inner enamel epithelium, laCL – labial cervical loop, liCL – lingual cervical loop, od – odontoblasts, oee – outer enamel epithelium, pa – preameloblasts, si – stratum intermedium, sr - stellate reticulum.

At E18.5 Fam20a expression in mouse incisor strongly correlates with the differential status of both the ameloblastic and odontoblastic lineage (Fig. 6.6). In mesenchymal cells *Fam20a* expression starts in early preodontoblasts getting stronger with the differentiation to odontoblasts. In epithelial cells expression is at lower level, however follows the same pattern. *Fam20a* expression is also observed at a low level in the labial CL area. The expression pattern of *Fam20a* in developing ameloblasts and odontoblasts was investigated further on P0 incisors (Fig. 6.7). Expression in preodontoblasts starts earlier then in preameloblasts, additionally *Fam20a* is expressed stronger in mesenchymal cells than in the epithelial. In epithelial cells ISH signal is strong in preameloblasts and ameloblasts at early secretory stage, getting lower in secreting ameloblasts after the production of the first enamel layer.

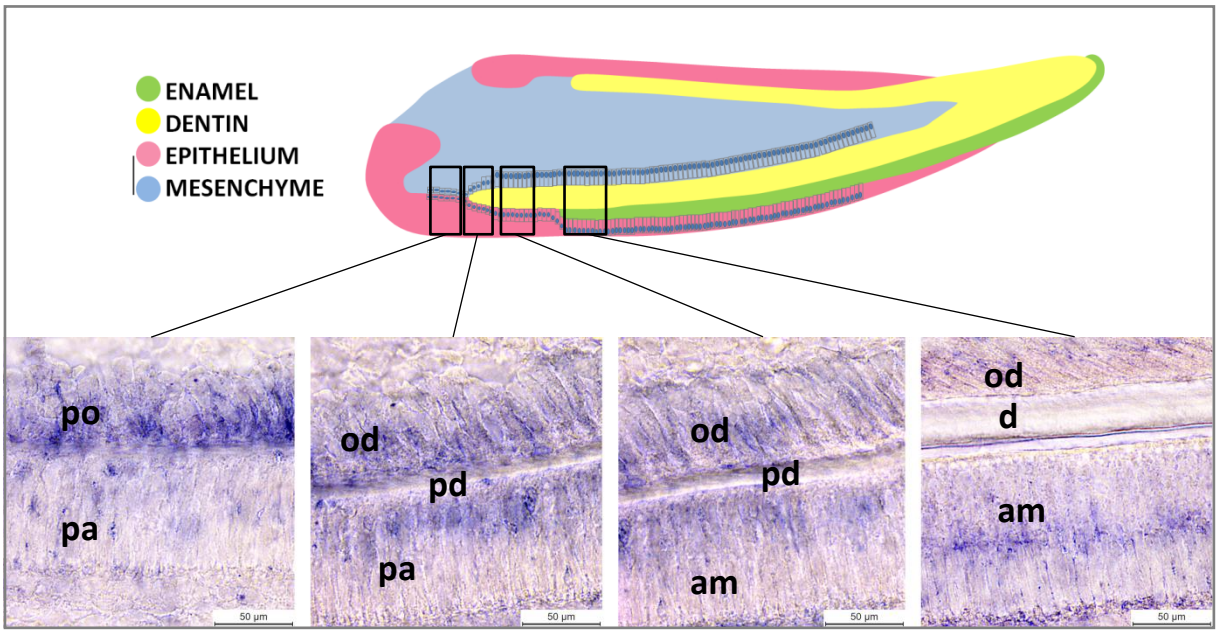


Figure 6.7. *In situ* hybridization showing the expression of *Fam20a* gene in a mouse incisor at P0. Abbreviations: am – ameloblasts, d – dentin, od – odontoblasts, pa – preameloblasts, pd – predentin, po – preodontoblasts.

Expression of Fam20a protein during orofacial development

Based on the ISH data, we analyzed the expression of the Fam20a protein at different developmental stages by immunohistochemical (IHC) analysis. Results for IHC in orofacial tissues during the embryonic development support the ISH results (Fig. 6.8). Fam20a was detected in the developing eye, nasal cavities, brain, skin, and bone.

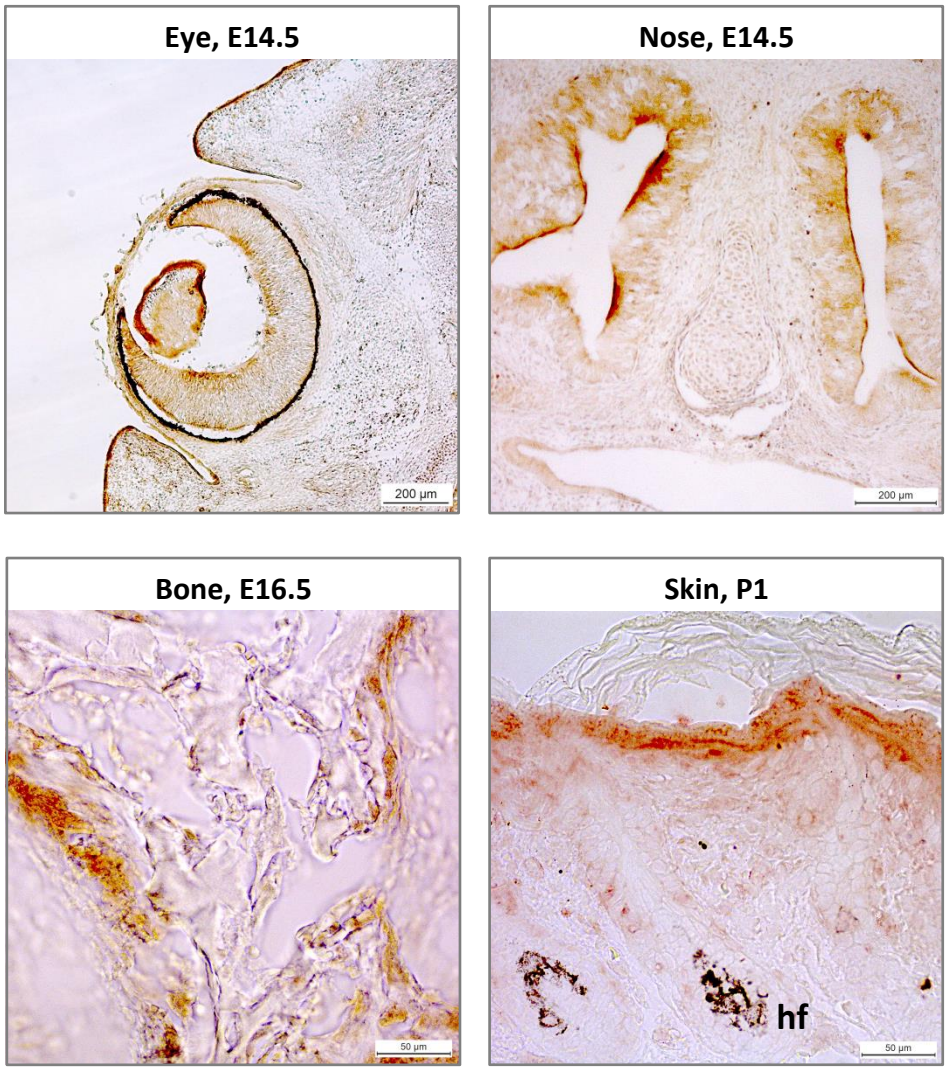


Figure 6.8. Immunohistochemistry showing Fam20a protein expression in developing mouse head. Abbreviations: hf – hair follicle.

In the early stages of tooth development the results of IHC were consistent with the results of ISH (Fig. 6.9, 6.10). At E14.5, Fam20a is localized in epithelial tissues of the tooth and also in the oral epithelium. At E17.5 Fam20a localization is more specific with the highest level in the least differentiated part, as in ISH, i.e., the CL areas. Moreover, Fam20a protein is expressed in mesenchymal tissues, especially in the cells close to the tooth epithelium.

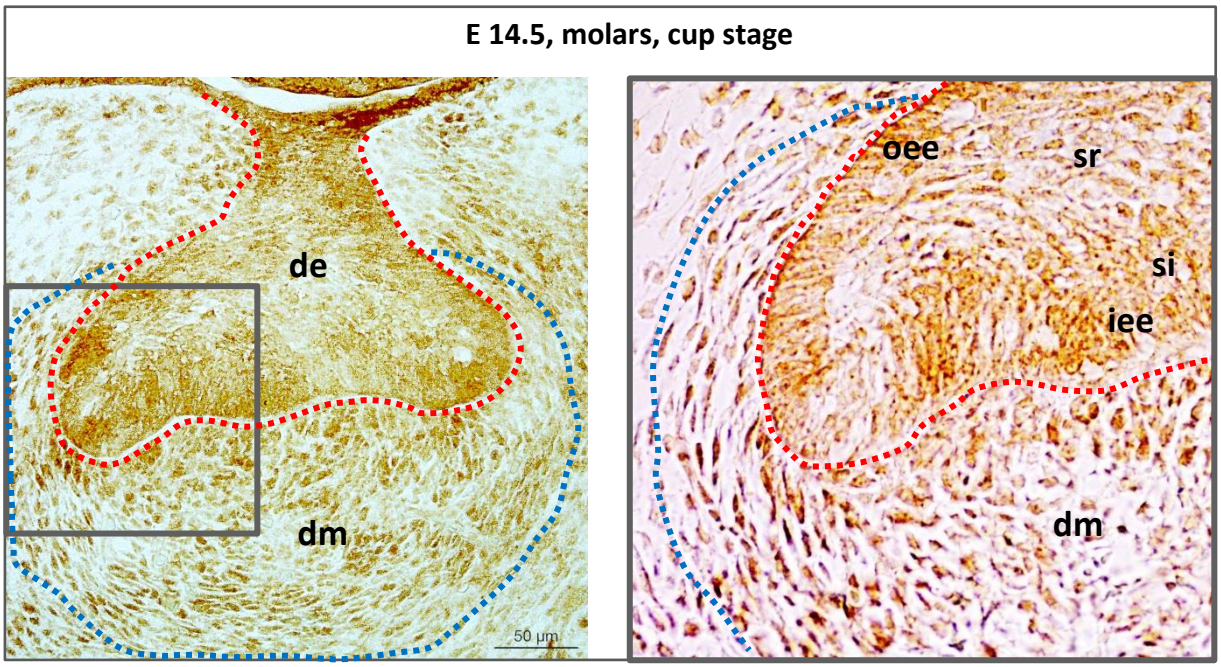


Figure 6.9. Immunohistochemistry showing Fam20a protein expression in a mouse molar at E 14.5. IHC. Abbreviations: de – dental epithelium, dp – dental papilla, dm – dental mesenchyme, iee – inner enamel epithelium, oee – outer enamel epithelium, pa – preameloblasts, po – preodontoblasts, si – stratum intermedium. sr - stellate reticulum .

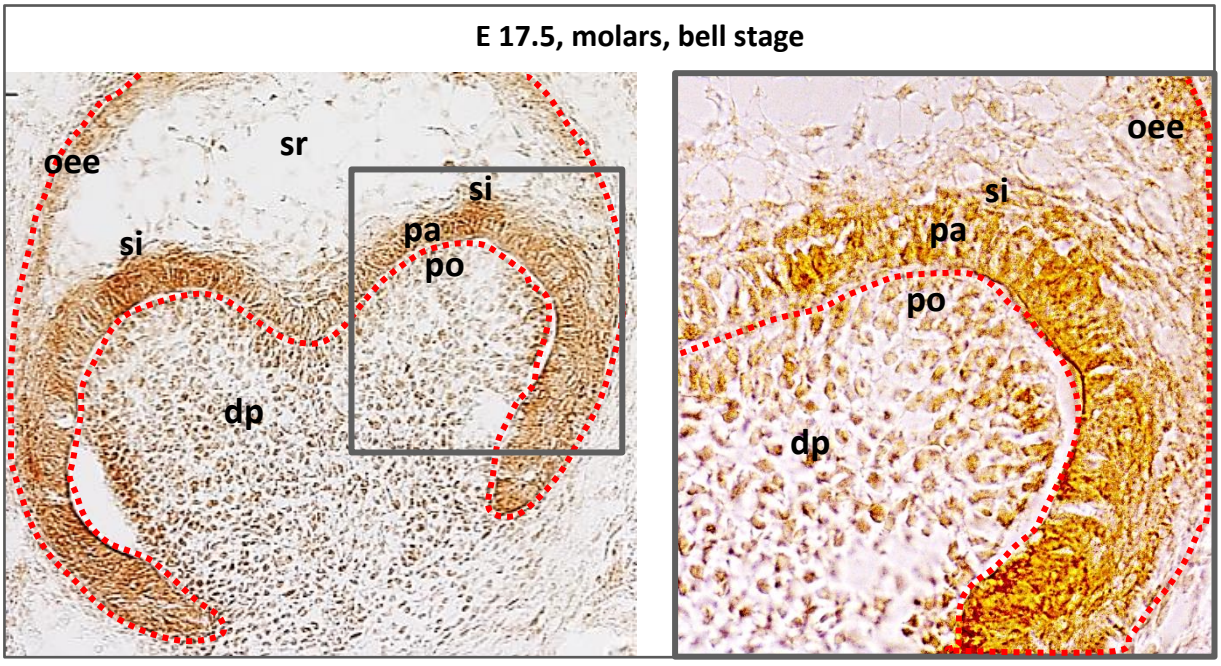


Figure 6.10. Immunohistochemistry showing Fam20a protein expression in a mouse molar at E 17.5. IHC. Abbreviations: de – dental epithelium, dp – dental papilla, dm – dental mesenchyme, iee – inner enamel epithelium, oee – outer enamel epithelium, pa – preameloblasts, po – preodontoblasts, si – stratum intermedium, sr - stellate reticulum .

In the Figure 6.11 the localization of Fam20a in the molars of newborn mice is shown. Mice have three molars which develop sequentially. The first molar develops first, the second molar develops second, and the third molar develops the last. Fam20a is strongly expressed in less differentiated areas such as the third molar and the CL areas of 1st and 2nd molars. In contrast to ISH, Fam20a protein is mostly detected in the ameloblastic lineage, mainly in preameloblasts and ameloblasts, and less in odontoblasts.

Using another anti-Fam20a antibody, we showed that Fam20a is a secreted protein (Fig. 6.12). During the secretory stage of amelogenesis, Fam20a is mostly localized at the apical secretory end of ameloblasts and in the layer of forming enamel. Fam20a expression was also detected in the cell body of ameloblasts, but at a lower level.

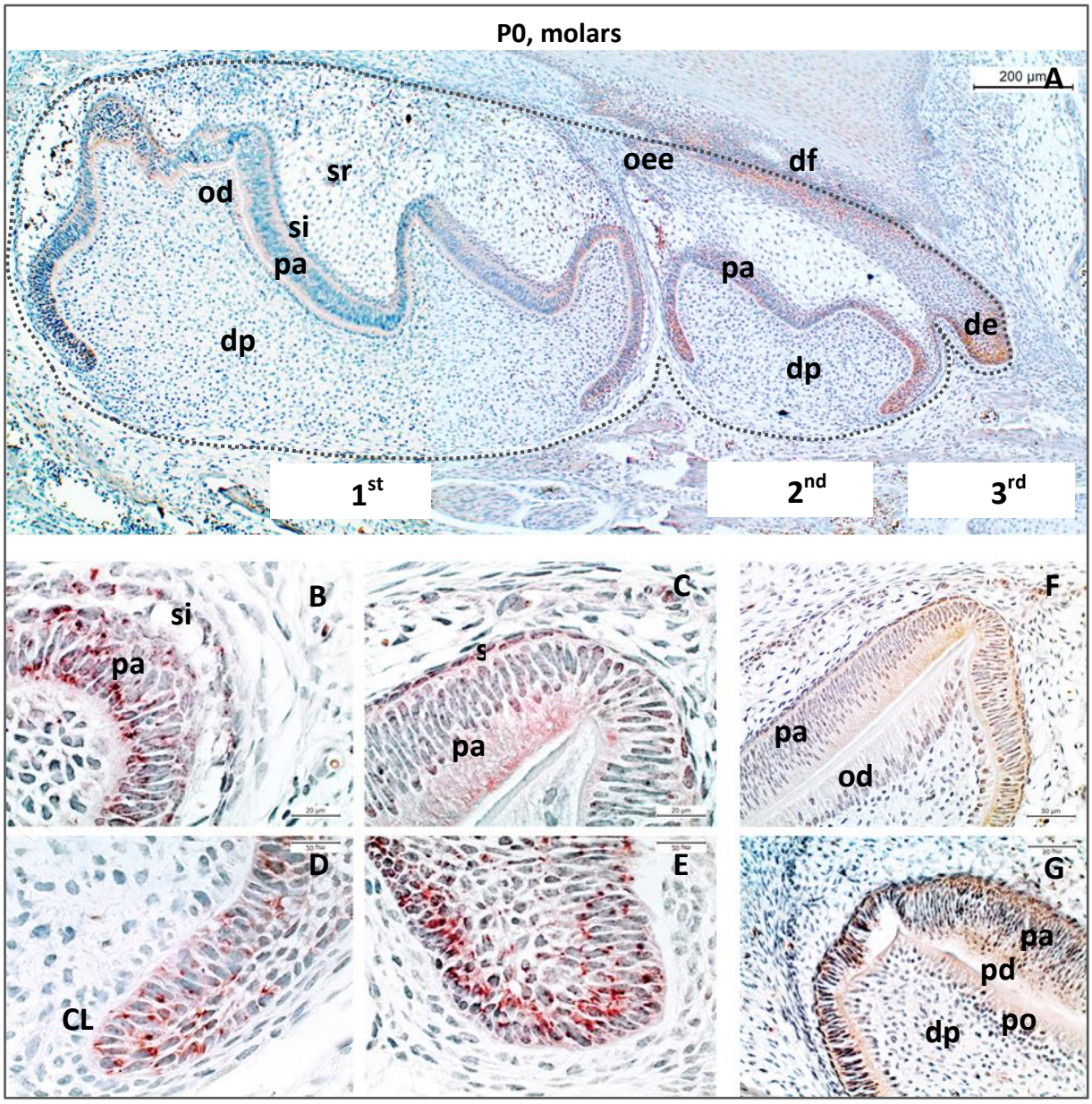


Figure 6.11. Immunohistochemistry showing Fam20a protein expression in the mouse molar at P0: (A) – overview of mouse molars, (B-G) – higher magnifications. Abbreviations: CL – cervical loop, de – dental epithelium, df – dental follicle, dp – dental pulp, de – dental epithelium, od – odontoblasts, oee – outer enamel epithelium, pa – preameloblasts, pd – predentin, po – preodontoblasts, si – stratum intermedium, sr - stellate reticulum.Dots mark tooth territory.

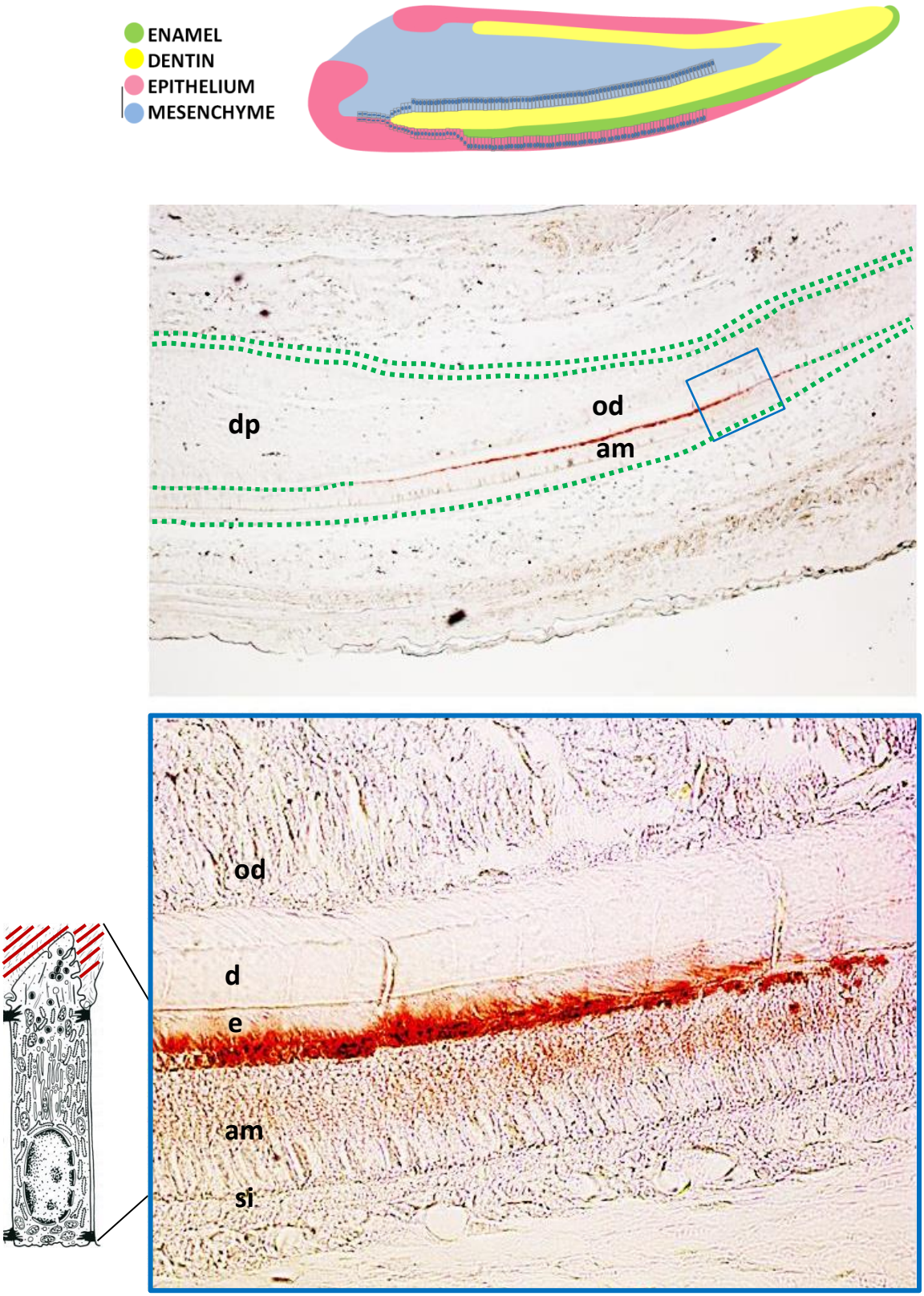


Figure 6.12. Immunohistochemistry showing Fam20a protein expression in ameloblasts and enamel of a P0 mouse incisor. Abbreviations: am – ameloblasts, d – dentin, dp – dental pulp, e – enamel, od – odontoblasts, si – stratum intermedium. Green dots mark epithelium of the incisor.

Fam20a mutant mice

In order to investigate the role of *Fam20a* in tooth development we used a transgenic mouse line where the first exon and a part of the first intron of *Fam20a* gene are replaced with the LacZ cassette.

Lac-Z staining was performed on the P0 Fam20a$^{+/-}$ mouse incisor and showed that it is expressed at the highest level in odontoblasts, lower in preodontoblasts and ameloblasts (Fig. 6.15). IHC staining against Fam20a on the LacZ stained incisor shows relative expression of Fam20a protein and gene (Fig. 6.14). While preodontoblasts, odontoblasts, and at lower levels also preameloblasts are LacZ positive, Fam20a protein is mostly located in the epithelial layer of the incisor: in the labial cervical loop and preameloblasts and only a bit in mesenchymal derived preodontoblasts.

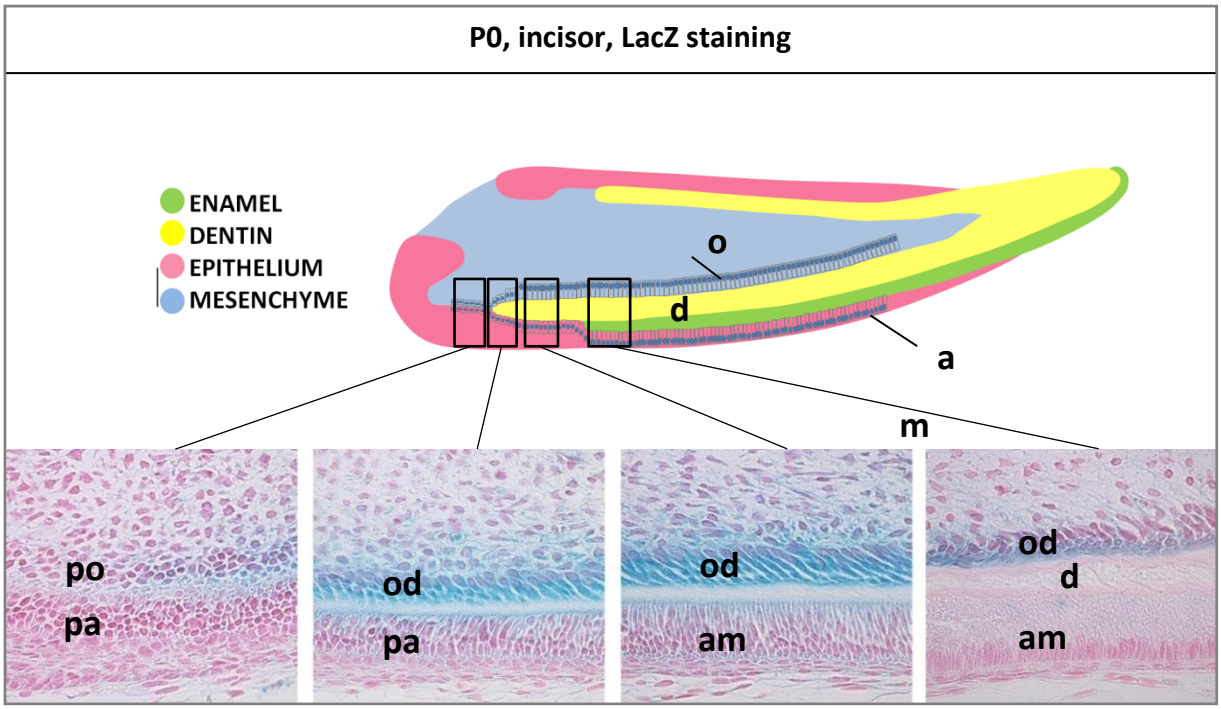


Figure 6.13. LacZ staining in the incisor of a *Fam20a*$^{+/-}$ mouse. Abbreviations: am – ameloblasts, d – dentin, od – odontoblasts, pa – preameloblasts, po - preodontoblasts.

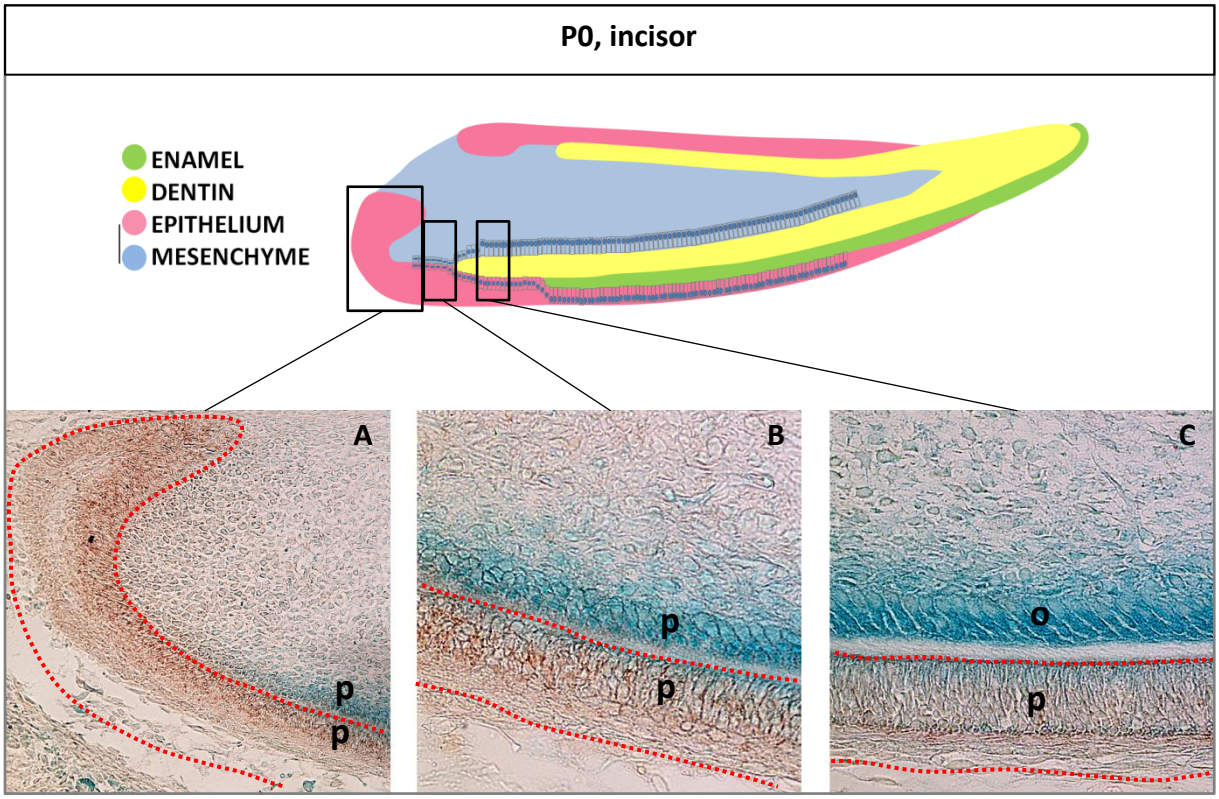


Figure 6.14. LacZ staining and immunohistochemistry against Fam20a protein in the incisor of a *Fam20a*$^{+/-}$ mouse. Abbreviations: od – odontoblasts, pa – preameloblasts, po - preodontoblasts.

Phenotypical analysis of *Fam20a* mutant mice

Comparisons of incisors of *Fam20a* mutants versus control wt mice showed that incisors exhibit defective enamel (Fig. 6.15). *Fam20a* mutant mice exhibit very white teeth in comparison with the wt control. Wt mouse incisors have a brownish color, which is the color of healthy enamel. The white color comes from the underlying dentin layer. Thus, the white color of the tooth means that the enamel is transparent, thin or does not exist that is why dentin is visible.

Both upper and lower molars of 3.5 months old wt and *Fam20a* mutant mice are presented in the Figure 6.16. The teeth of the *Fam20a* mutant mice are smaller than the teeth of wt mice. Moreover, in comparison with the sharp and clearly defined cusps of the wt mouse molars, the molars of the *Fam20a* mutants are flat. Smaller teeth and a flat surface could be a developmental problem or a result of tooth destruction.

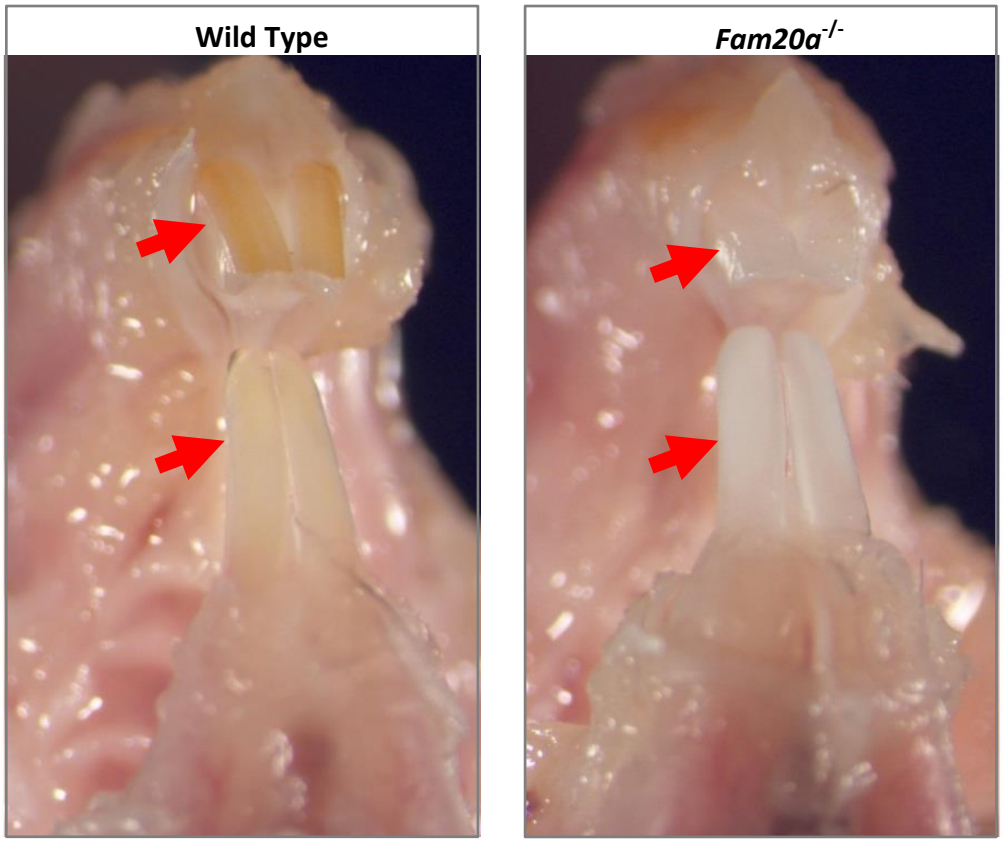


Figure 6.15. Incisors of 3.5 months old wild type and Fam20a$^{-/-}$ mice. Red arrows point at the mouse incisors.

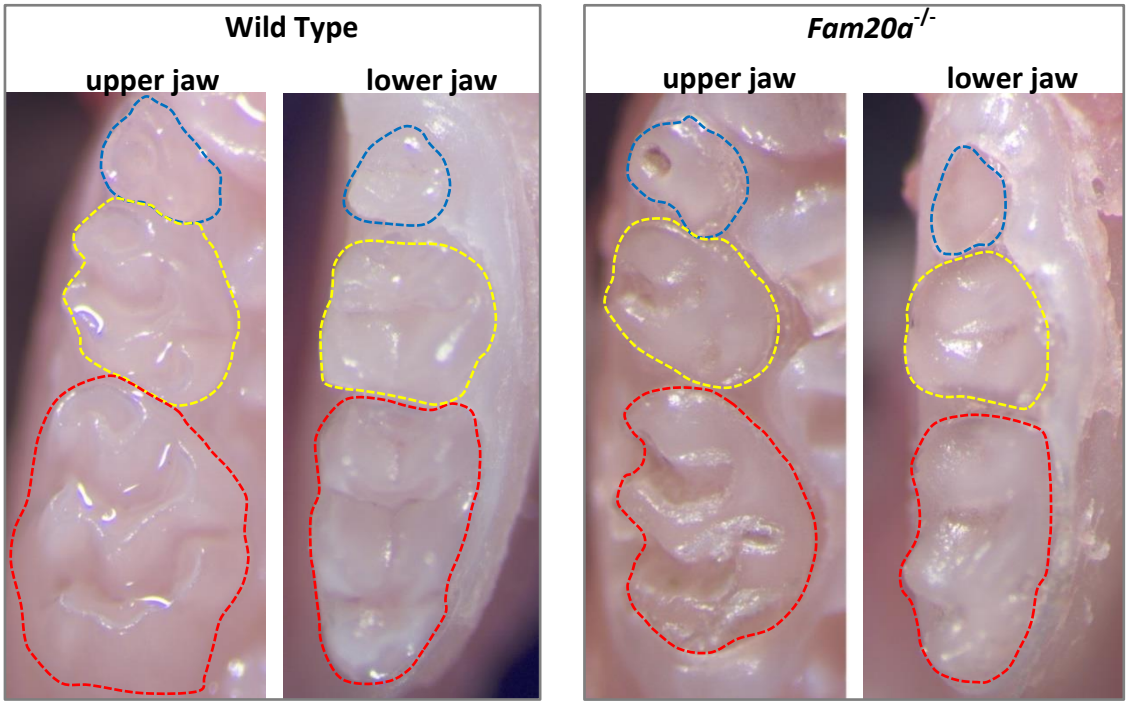


Figure 6.16. Molars of 3.5 months old wild type and Fam20a$^{-/-}$ mice. Red dots mark the 1st molar, yellow dots mark the 2nd molar, and blue dots mark the 3rd molar.

Following these observations, we performed µCT analysis in order to staudy in detail enamel defect observed. In mouse incisors of the control mouse the enamel appears as a white layer (Fig. 6.17). Very bright white enamel indicates high mineral content. This layer is clearly defined and covers the underlying layer of dentin. In comparison, *Fam20a* mutants lack this clearly defined highly mineralized layer. Instead, they have some mineralized material, which covers the tooth. This material is very thin, disorganized, detached from the dentin and disappears shortly after tooth eruption. The tooth and enamel in particular falls under significant pressure during mastication. If the enamel defect is severe, as is the case in *Fam20a* mutants, it will not be able to handle it and will eventually sooner or later wear out until complete extinction. The defective structure together with the low mineralization of the enamel in *Fam20a* mutants leads to the loss of the enamel soon after the eruption. This could be also observed in the molars (Fig. 6.18). Molars of the *Fam20a* mutant adult mice lose their enamel completely. Presumably after the enamel loss the outer layer of dentin, which is not predisposed to have a direct contact with food and components of the oral cavity, starts to be lost.

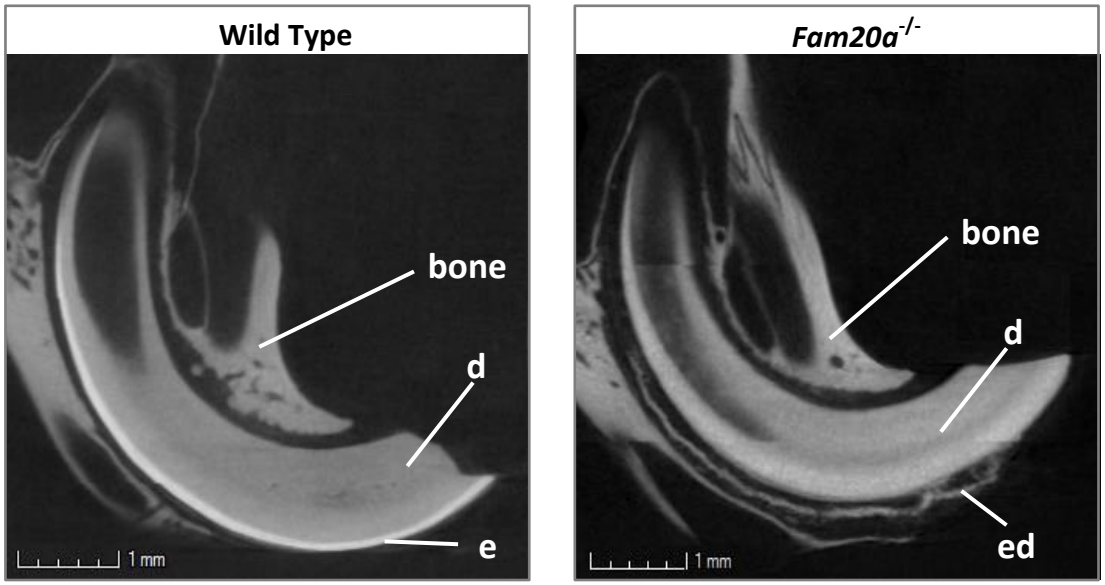


Figure 6.17. µCT analysis of the incisors of 3.5 months old mice. Abbreviations: d – dentin, e – enamel, ed - enameloid.

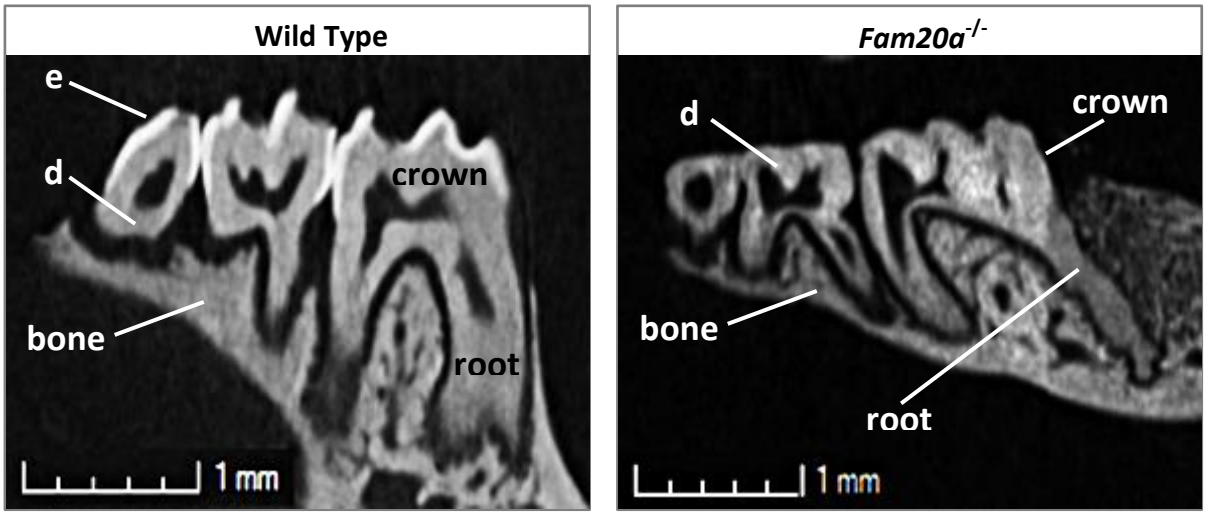


Figure 6.18. µCT analysis of the molars of 3.5 months old mice. Abbreviations: d – dentin, e – enamel.

Histological analysis of *Fam20a* mutant mice

To investigate at which stage of enamel development the observed defect appears we performed histological analysis of the incisors of new born mice (Fig. 6.19). The development of enamel producing ameloblasts goes through several stages, which can be visualized through the change in shape and size of these cells: starting from a square shape to more elongated column to highly elongated and polarized cells. By the time ameloblasts start producing enamel, they have formed a layer of elongated columnar cells, which are in close contact with each other. Enamel secreting ameloblasts are polarized cells: they have an apical part where secretion takes place and a basal part, where the nucleus of a cell is located. From our results, we noticed that defects of the enamel in *Fam20a* mutants start during the development. Everything proceeds normally until the ameloblasts start producing enamel. Shortly after the production of the first enamel layer, ameloblasts detach from their enamel layer, lose their polarity and disorganize. Mineralized material is secreted continuously, but not in an organized fashion as a proper enamel layer.

Incisors of the adult *Fam20*$^{-/-}$ mouse show the same. Ameloblasts detach and disorganize. Only thin layer of enamel like material – enameloid - is attached to the dentin (Fig. 6.20). Interestingly, these disorganized ameloblasts keep producing mineralized nodules that are deposited between the cells (Fig. 6.21).

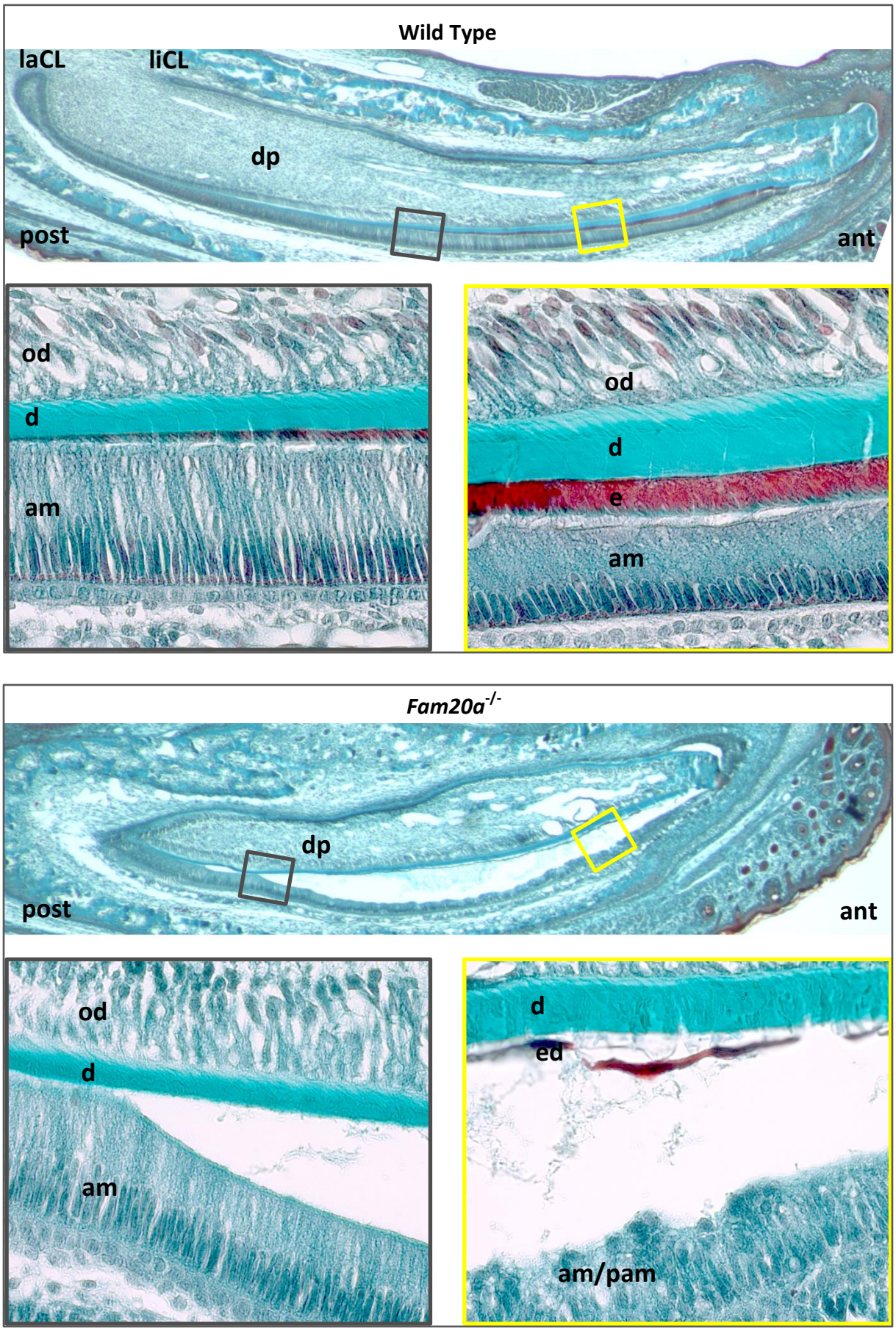


Figure 6.19. Histological analysis of P0 incisors. Abbreviations: am – ameloblasts, ant – anterior part, d – dentin, dp – dental pulp, e – enamel, ed – enameloid, od – odontoblasts, pa – preameloblasts, post – posterior part.

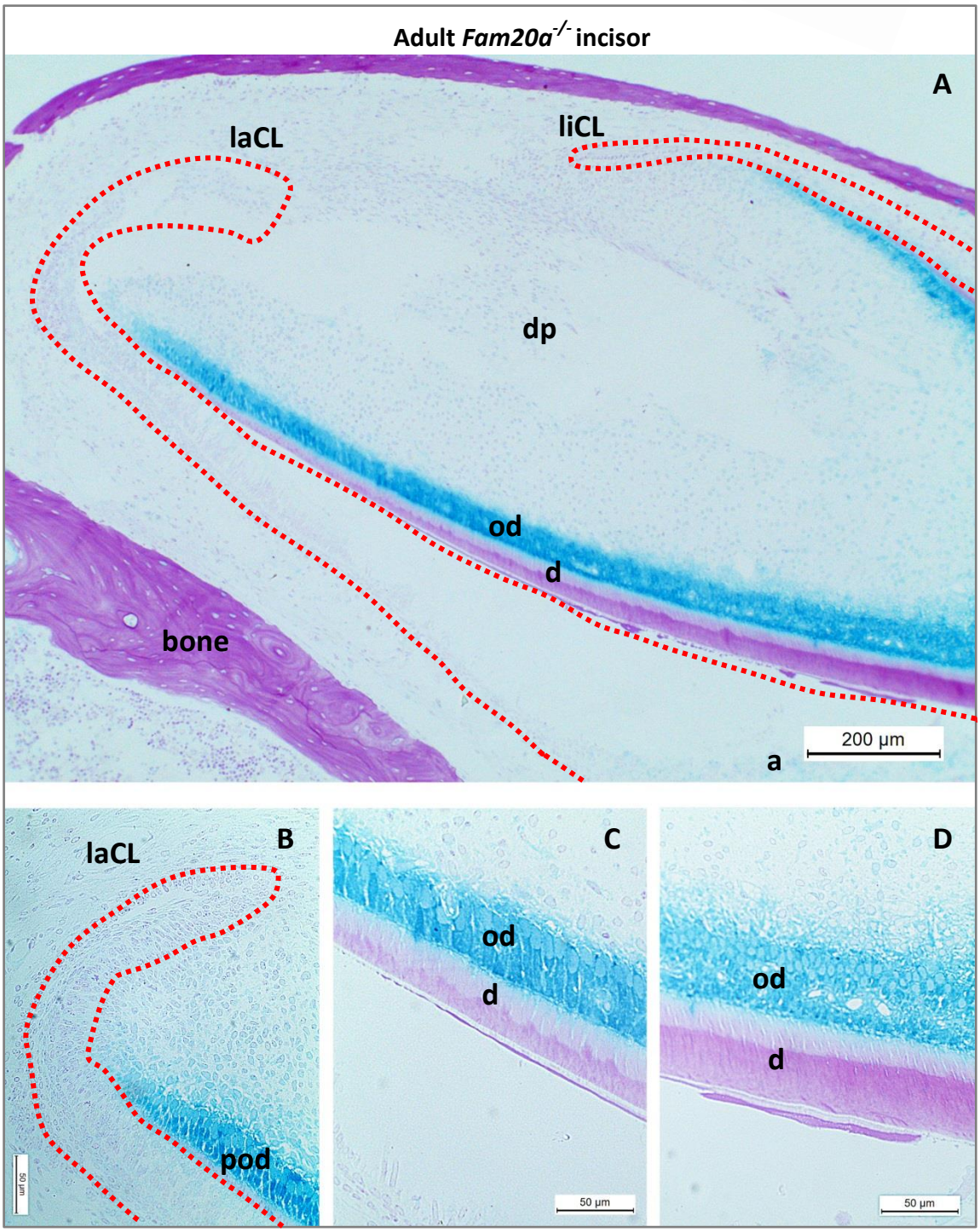


Figure 6.20. LacZ staining of the adult Fam20a$^{-/-}$ mouse incisor. Abbreviations: am – ameloblasts, d – dentin, dp – dental pulp, laCL – labial cervical loop, liCL – lingual cervical loop, od – odontoblasts, po - preodontoblasts (Pictures by Prof. Luder).

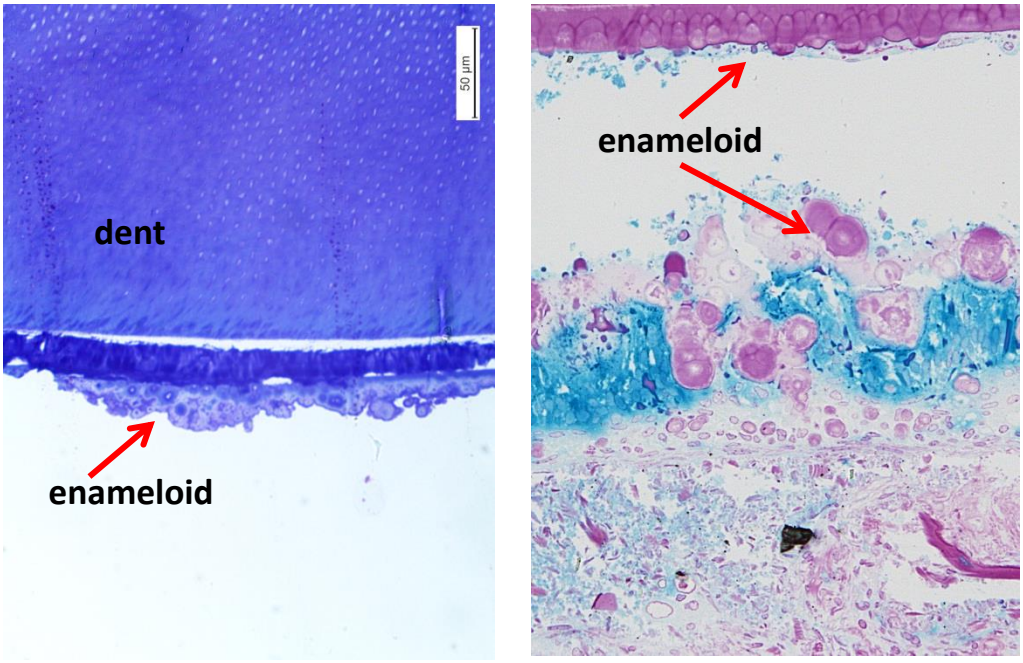


Figure 6.21. Formation of enameloid in *Fam20a*$^{-/-}$ mice (Pictures by Prof. Luder)

As we have shown before, molars of the adult *Fam20a*$^{-/-}$ mice exhibit enamel defects that lead to the full loss of enamel after the eruption. Figure 6.22 shows the molar of *Fam20a*$^{-/-}$ mouse just before the eruption is shown. It is covered by the same enameloid material, as the unerupted part of the incisor. Moreover, the LacZ staining shows that Fam20a is also expressed in gum epithelium between molars.

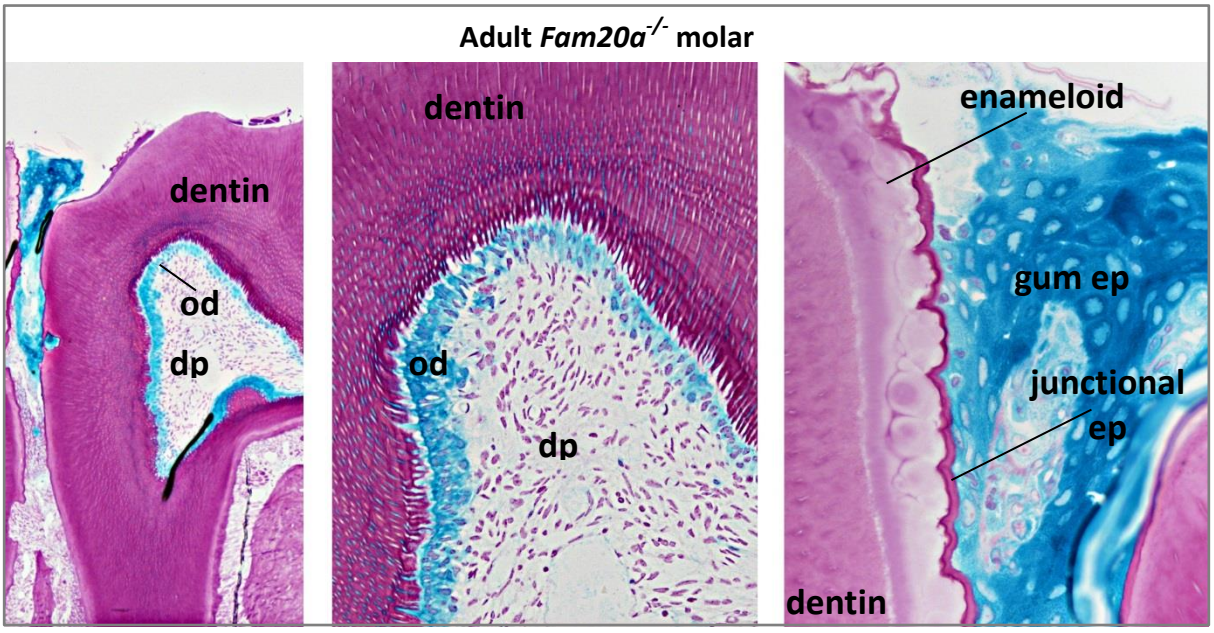


Figure 6.22. LacZ staining of the adult Fam20a$^{-/-}$ mouse molar. Abbreviations: dp – dental pulp, ep – epithelium, od – odontoblasts (Pictures by Prof. Luder).

Analysis of Fam20a$^{-/-}$ mouse teeth by backscattered electron microscopy

In order to investigate in deeper detail the structure of the material formed in Fam20a-/- mice, we performed backscattered electron microscopy (BEM). BEM of incisors of adult mice showed the structure that is formed in *Fam20a* mutants instead of normal enamel (Fig. 6.23). This structure could be divided into two parts: a layer attached to the dentin and a mass of chaotic mineralized nodules. Both structures have low mineral content and poor organization. Erupting molars of Fam20a$^{-/-}$ mice have only a thin layer of enameloid that covers only some parts of the tooth (Fig. 6.24).

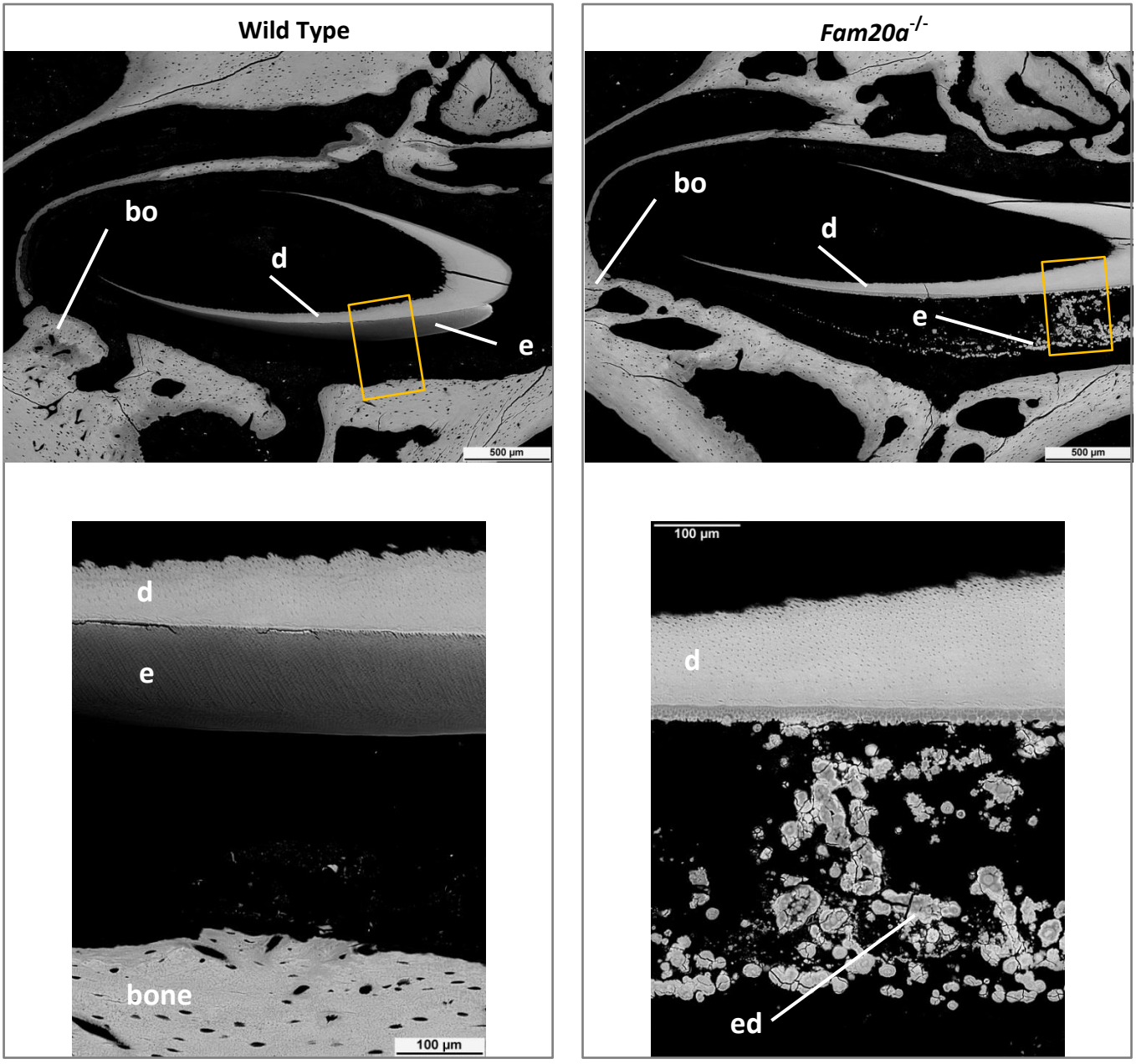


Figure 6.23. Backscattered electron microscopy of molars of adult wildtype and Fam20a$^{-/-}$ mice. Structure of enamel and dentin. Abbreviations: d –dentin, e – enamel, ed – enameloid (Pictures by Prof. Luder).

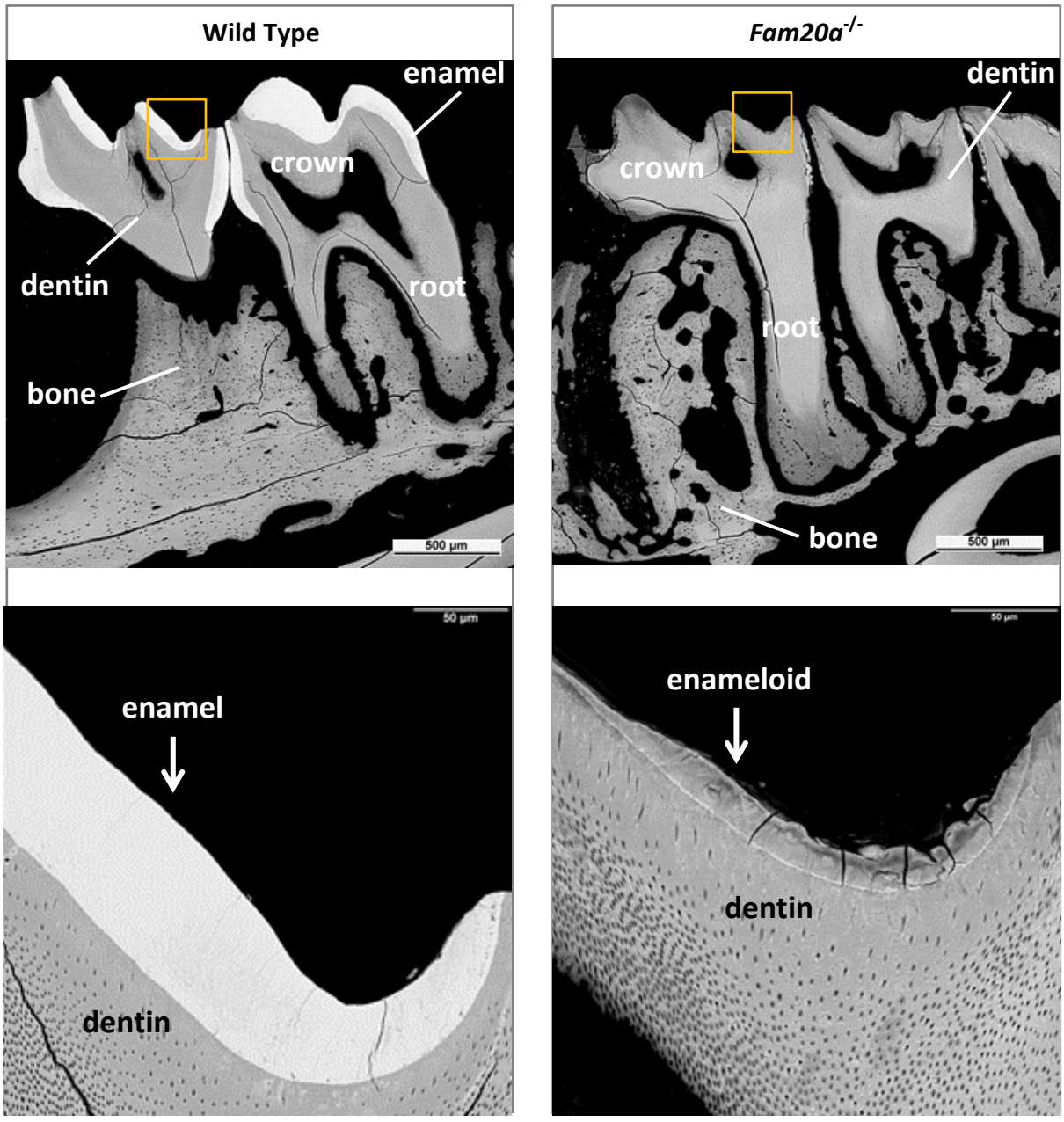
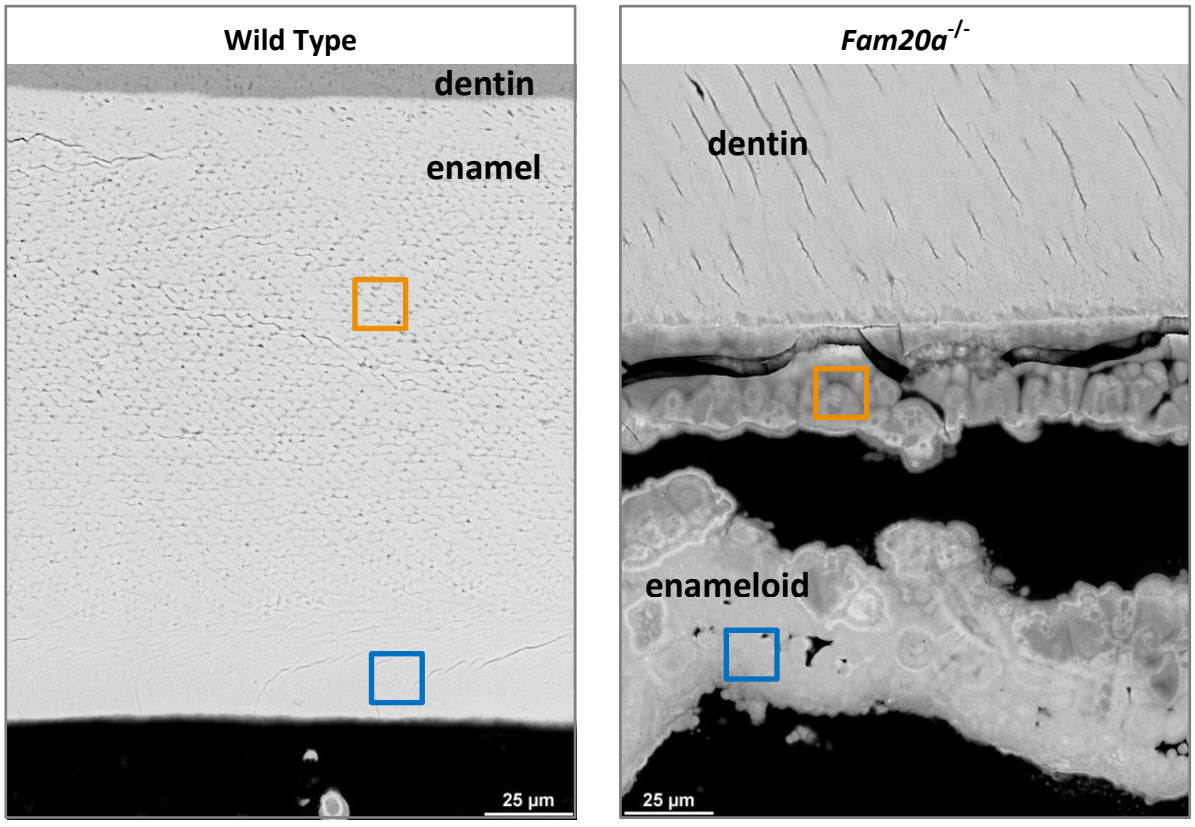


Figure 6.24. Backscattered electron microscopy of molars of adult mice. Structure of enamel and dentin (Pictures by Prof. Luder).

To analyze enameloid that is formed in *Fam20a*$^{-/-}$ teeth we performed an elemental analysis (Fig. 6.25). It showed that indeed in *Fam20a* mutants two types of material are formed instead of normal enamel. The first, which is attached to the dentin, has higher concentrations of magnesium (Mg) and sulfur (S) in comparison with the enamel of a wt mouse. The content of mineralized nodules also differs from the wt enamel. While the ratio of calcium/phosphorus (Ca/P) is higher than in wt teeth, it also exhibits higher contents of Mg and S, and a lower content of Fe compared to the wt enamel.



Element	Wild Type	*Fam20a*$^{-/-}$
Ca/P	1.72	1.72
Mg	0.16	0.51
S	0.00	0.30
Ca/P	1.72	2.20
Mg	0.00	0.30
S	0.00	0.86
Fe	0.26	0.00

Figure 6.25. Backscattered electron microscopy of molars of adult mice (3.5 months old). Structure of enamel and dentin (Pictures by Prof. Luder).

Expression of main genes involved in enamel development

The expression of genes involved in enamel development was evaluated in wt and *Fam20a*$^{-/-}$ mouse teeth. To achieve this, we performed ISH of *Amb*, *Amg*, *Mmp20,* and *Dspp* on P0 incisors (Fig. 6.26). Localization and the level of expression of all these genes appeared unchanged in *Fam20a* mutants in comparison with the control.

We also investigated the localization of one of the main enamel proteins – Amg in P0 incisors (Fig. 6.27). The expression of Amg in mutant and control mice is restricted to preameloblasts, ameloblasts, partly to odontoblasts and the first layer of enamel. Additionally, enameloid that is formed in mutant teeth is highly enriched with Amg.

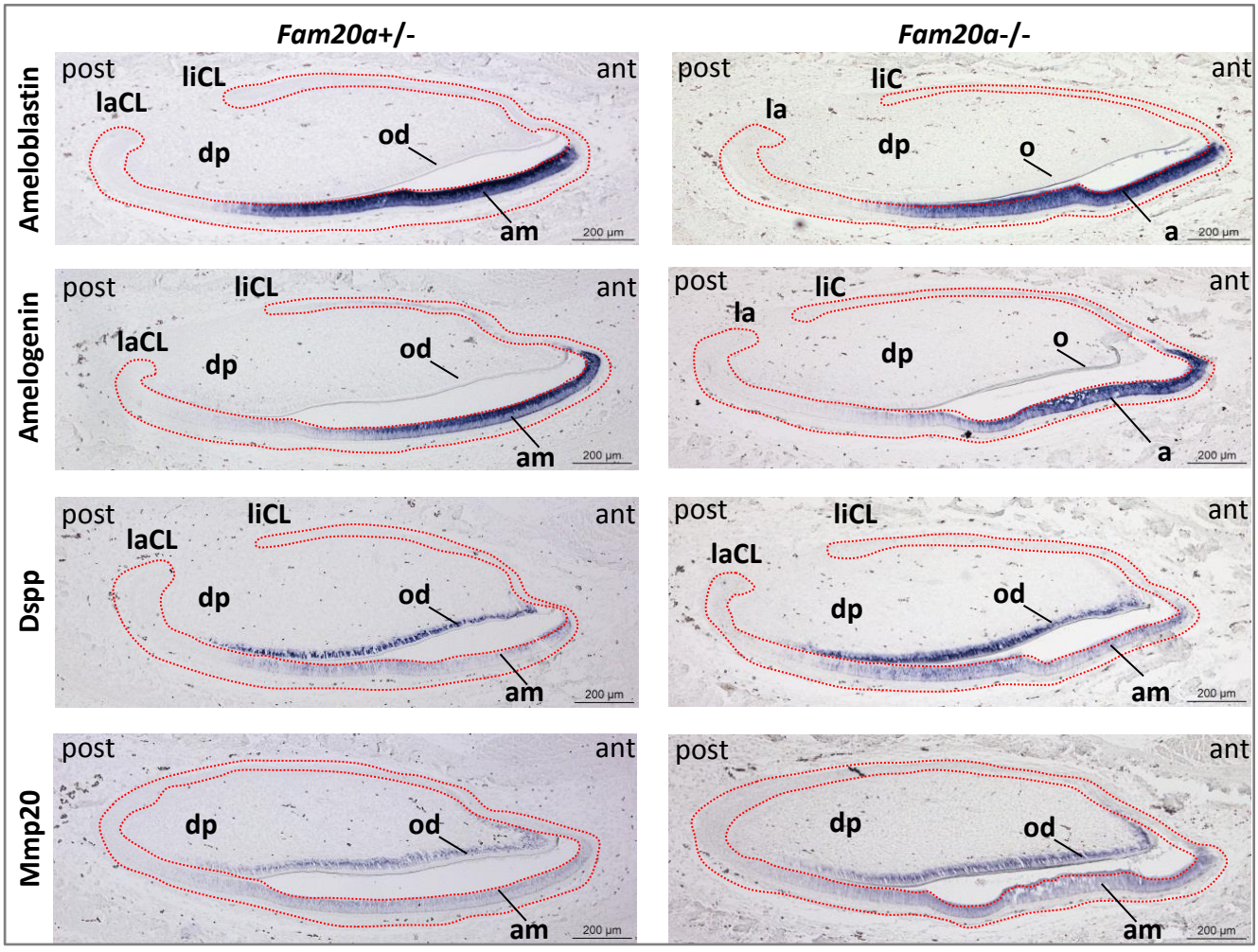


Figure 6.26. *In situ* hybridization showing the expression of genes associated with enamel development. Mouse incisor at P0. Abbreviations: am – ameloblasts, ant – anterior part, d – dentin, dp – dental pulp, ed – enameloid, laCL – labial cervical loop, liCL – lingual cervical loop, od – odontoblasts, post – posterior part.

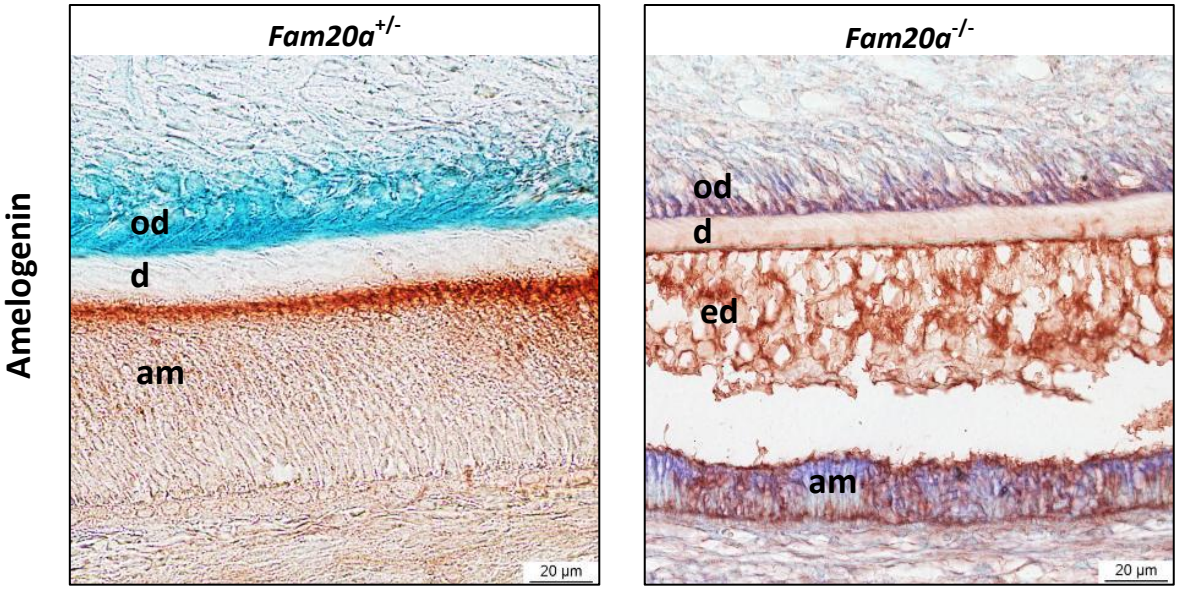


Figure 6.27. Expression of Amg protein in control and *Fam20a*$^{-/-}$ mouse incisors. Abbreviations: am – ameloblasts, d – dentin, ed – enameloid, od – odontoblasts.

Regulation of *Fam20a* expression by epithelial/mesenchymal interactions

The development of a tooth strongly depends on the interaction of two involved cell types: the epithelial and the mesenchymal cells. *Fam20a* is expressed in both of them during the development. Thus, we wanted to find the role of these tissues as well as the interaction between them in the development of enamel defects in *Fam20a* mutant mice. Therefore, we performed a recombination experiment (Fig. 6.28). The molars from *Fam20a*$^{-/-}$ and wt mice were dissected at E14.5, then the epithelium and the mesenchyme were separated (Fig. 6.28, A). After that they were recombined, resulting in four recombinant types (Fig. 6.28, B, Table 6.1).

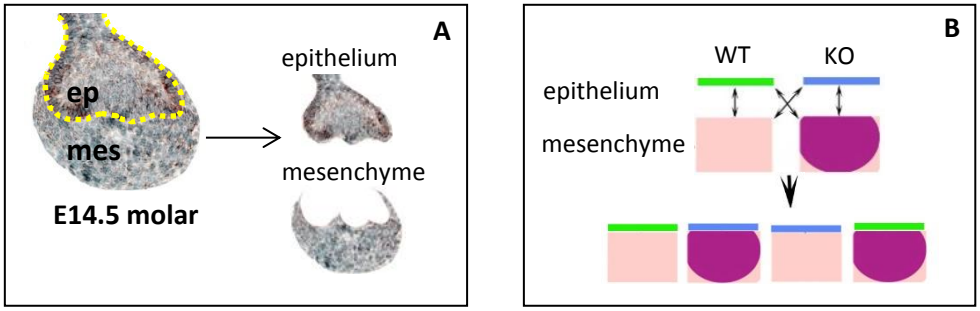


Figure 6.28. Scheme of the recombination experiment. Abbreviations: ep – epithelium, mes – mesenchyme, WT – wild type, KO – Fam20a mutant.

	Epithelium	Mesenchyme	Expected result
1	wt	mutant	
2	mutant	wt	
3	wt	wt	Normal tooth with good enamel
4	mutant	mutant	Tooth with enamel defect

Table 6.1. *Recombinants and expected outcome.*

Recombinants were cultured overnight at 37°C and then placed surgically under the kidney capsule of immunocompromised mice for one month. This system allows tooth growth *ex vivo*. After that kidneys were collected and the recombinants were analyzed. The result of the µCT analysis of the recombinants is presented in Figure 6.29.

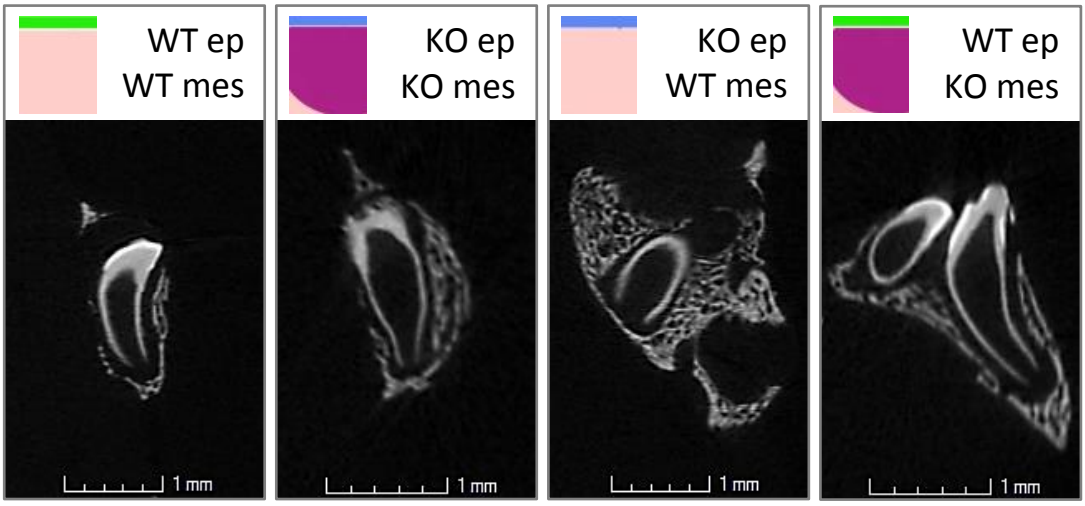


Figure 6.29. µCT analysis of recombinants after one month under the kidney capsule. Abbreviations: ep – epithelium, mes – mesenchyme, WT – wild type, KO – Fam20a mutant.

Both controls wt/wt and mutant/mutant showed the expected results. In the case of the mutant epithelium and wt mesenchyme obtained teeth had no enamel, indicating that the wt mesenchyme cannot lead appropriate enamel development in the presence of the *Fam20a* mutation in the epithelium. When wt epithelium was recombined with the mutant mesenchyme obtained teeth had enamel with the same mineralization level as in wt/wt control. Taken together, these results show that in *Fam20a* mutant mice, enamel defects are tissue autonomous - epithelial specific.

To prove the origin of the tissue for each recombinant, we performed anti beta galactosidase staining. *Fam20a* mutant has LacZ cassette, thus tissues taken from the mutant embryos will be positive for beta galactosidase activity, while tissues from the wt will be negative (Fig. 6.30, 6.31). For the histological analysis of the recombinants hematoxyline/eosin staining was performed (Fig. 6.30, 6.31). Recombinants, in which epithelium came from the wt mice have a normal structure of epithelium and it produces proper enamel. Epithelium from the *Fam20a*$^{-/-}$ mice leads to formation of a tooth with defective enamel - enameloid. Epithelial cells in these recombinants do not form normal ameloblasts; instead they form a layer of round disorganized cells. The enameloid has the same appearance as the one formed in *Fam20a* mutant mice *in vivo* and also has two parts: one between epithelial cells and dentin, and the other as mineralized nodules between epithelial cells.

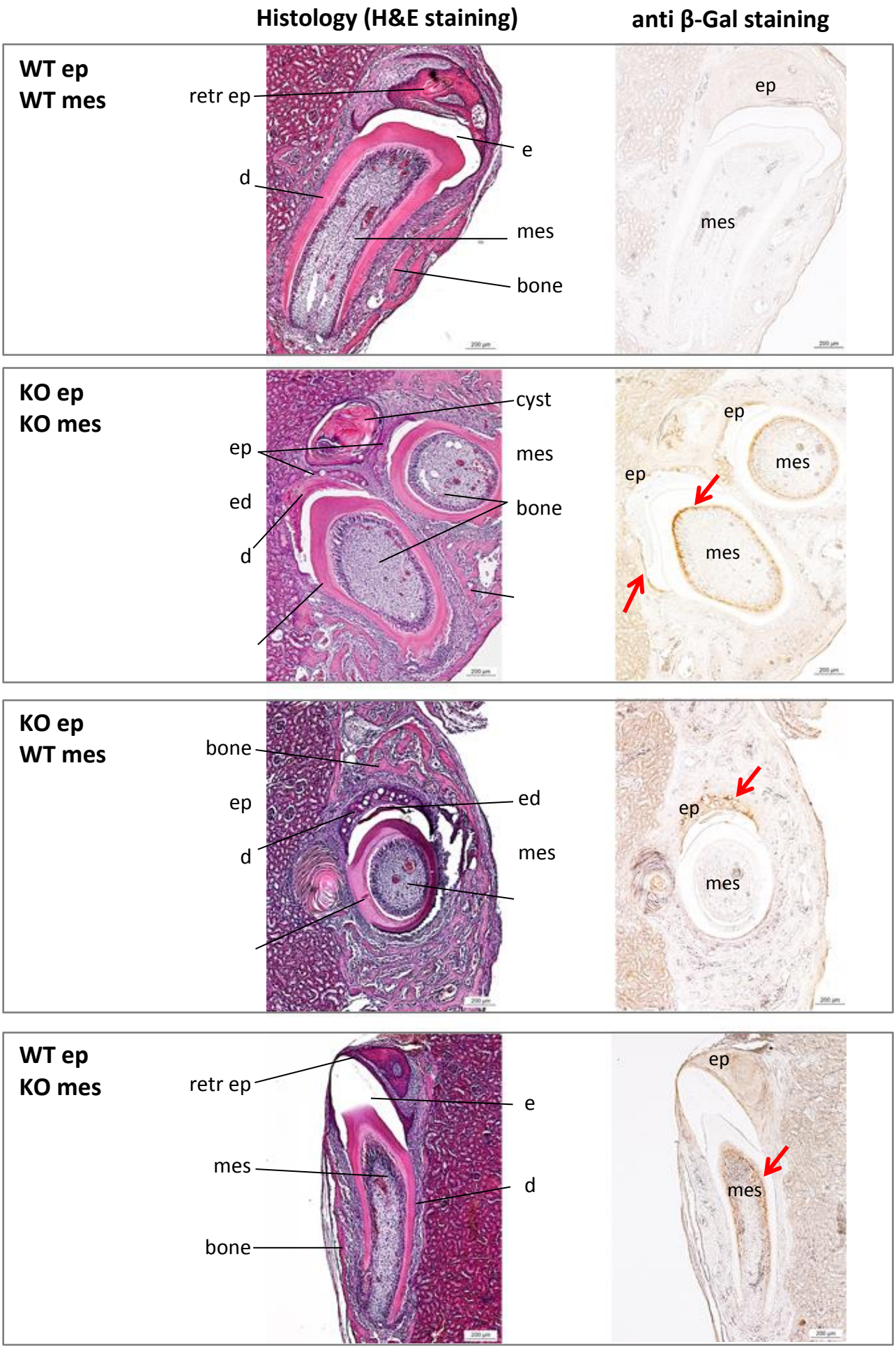


Figure 6.30. Histological analysis of recombinants by hematoxyline/eosin staining. Staining for beta galactosidase activity to distinguish the origin of the tissue. Abbreviations: e – enamel, ed – enameloid, d – dentin, ep – epithelium, mes – mesenchyme, retr ep – retracted epithelium.

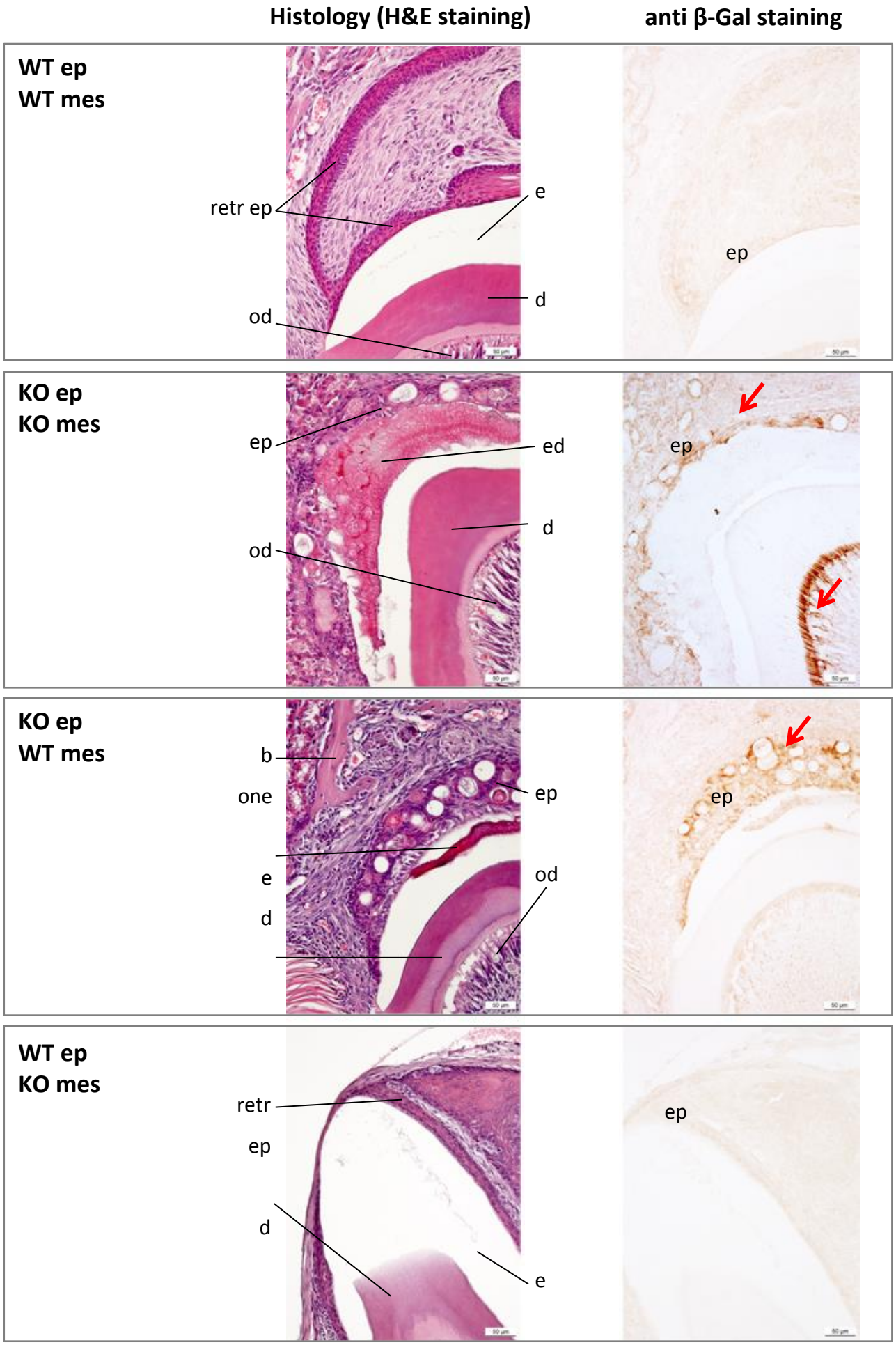


Figure 6.31 Histological analysis of the epithelium of recombinants by hematoxyline/eosin staining. Staining for beta galactosidase activity to distinguish the origin of the tissue. Abbreviations: e – enamel, ed – enameloid, d – dentin, ep – epithelium, mes – mesenchyme, retr ep – retracted epithelium.

Phenotype rescue experiment

The aim of our phenotype rescue experiments is to find a way to rescue the enamel defect in Fam20 mutant mice. To perform that we designed and produced recombinant FAM20A protein. First, we cloned the protein sequence in the pCEP4 vector together with the signal peptide for protein secretion and a His-tag for selection. Then we transfected HEK-EBNA cell line with the construct (Fig. 6.32).

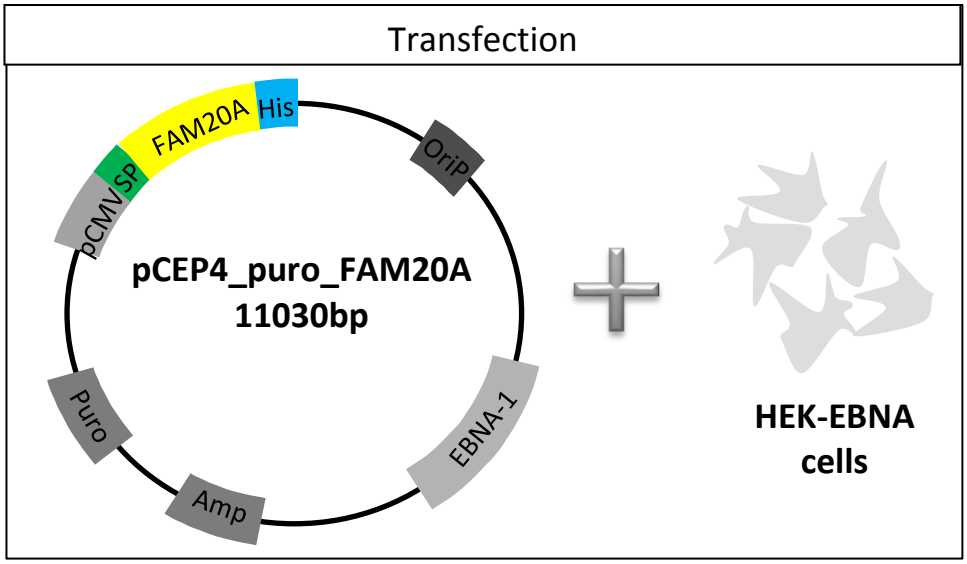


Figure 6.32. Protein production. Transfection. Abbreviations: pCMV – human cytomegalovirus promoter, SP – signal peptide, His – his tag, OriP - origin of replication, EBNA-1 - Epstein–Barr nuclear antigen 1, Amp – amplicilin, Puro – puromycin.

The efficiency of transfection was analyzed by immunofluorescence using anti-His antibody. Positive cells signified that we obtained a cell line producing FAM20A (Fig. 6.33).

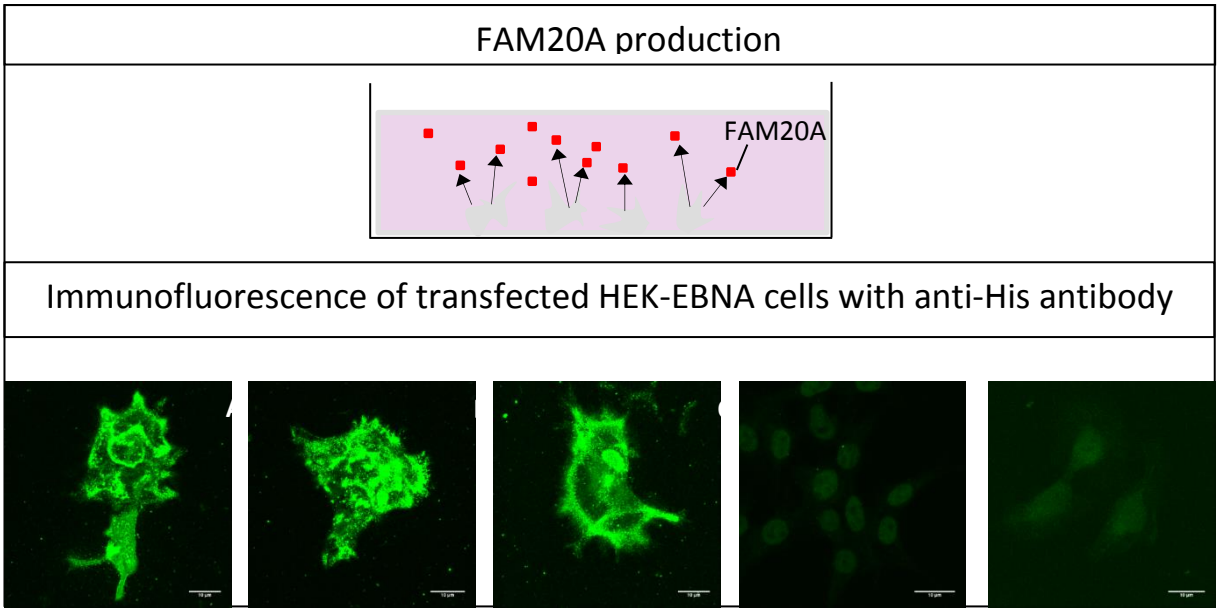


Figure 6.33 Protein production. Cell line after transfection. . Immunofluorescence of transfected HEK-EBNA cells with anti-His antibody: (A), (B), (C) – cells after transfection and immunohistochemistry; controls: (D) – nontransfected cells, (E) – no primary anti-bodies were used (Pictures by Dr Maria Mitsi, PSI).

Further analysis of the medium collected from the cell culture at different time points by Western blot showed that cells secret FAM20A (Fig. 6.34). This medium was further used for the phenotype rescue experiments. Concentration of FAM20A in the medium was 1mg/ml.

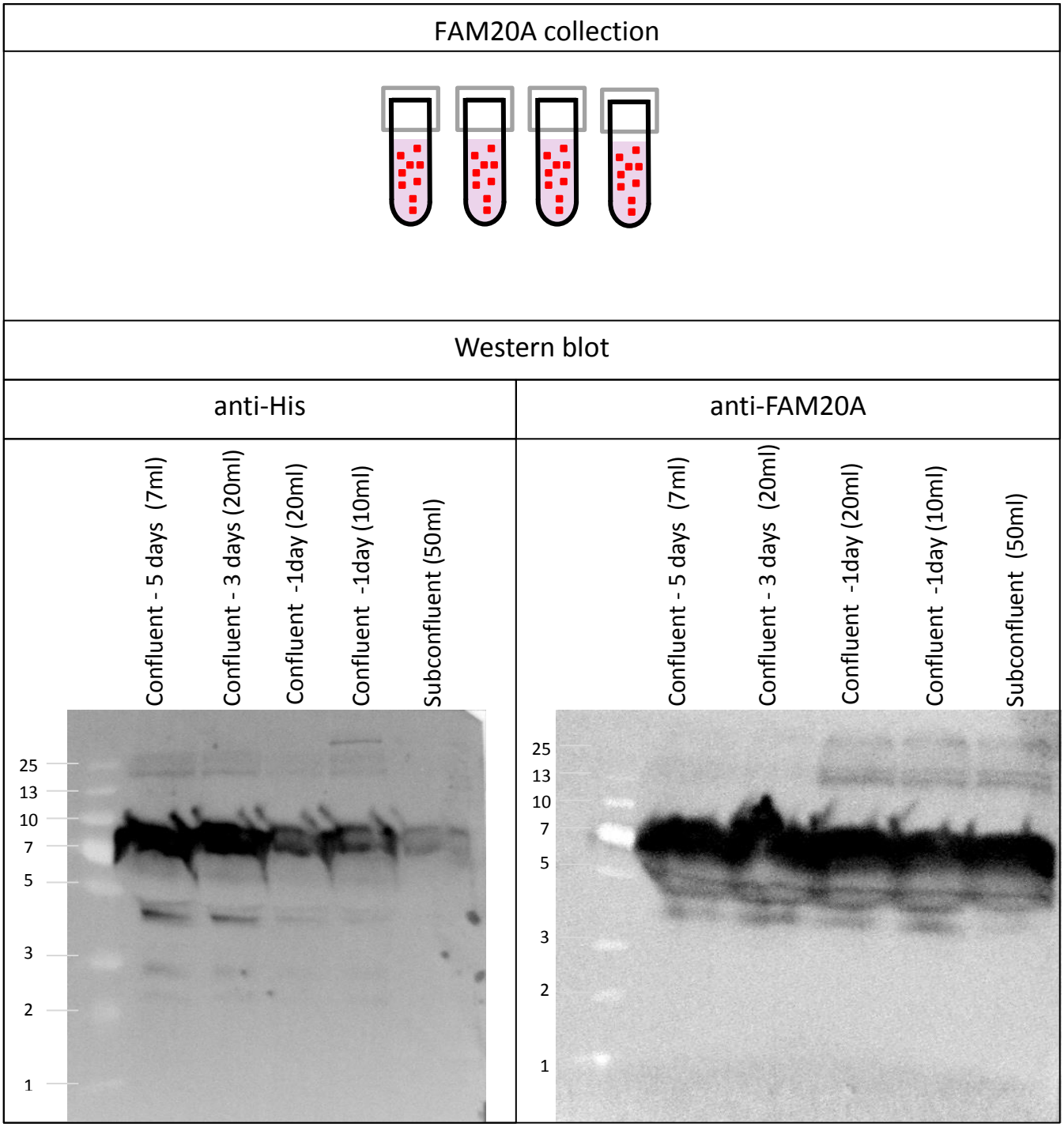


Figure 6.34. Analysis of the media from transfected HEK-EBNA cells by Western blot (Pictures by Dr Maria Mitsi, PSI).

In *Fam20a* mutant mice enamel defects appear at the stage of early enamel development. Therefore, we dissected incisors and molars of *Fam20a*$^{-/-}$ mice at E16.5 when enamel is not formed yet and cultured them on semi-solid plates for a week. During this week teeth received medium with the recombinant FAM20A protein at a concentration of 1 mg/ml twice a day (Fig. 6.35). Control teeth were cultured in the same medium without addition of the FAM20A protein. After one week the teeth were placed under the kidney capsule of immunocompromised mice to grow for three days and then they were analyzed.

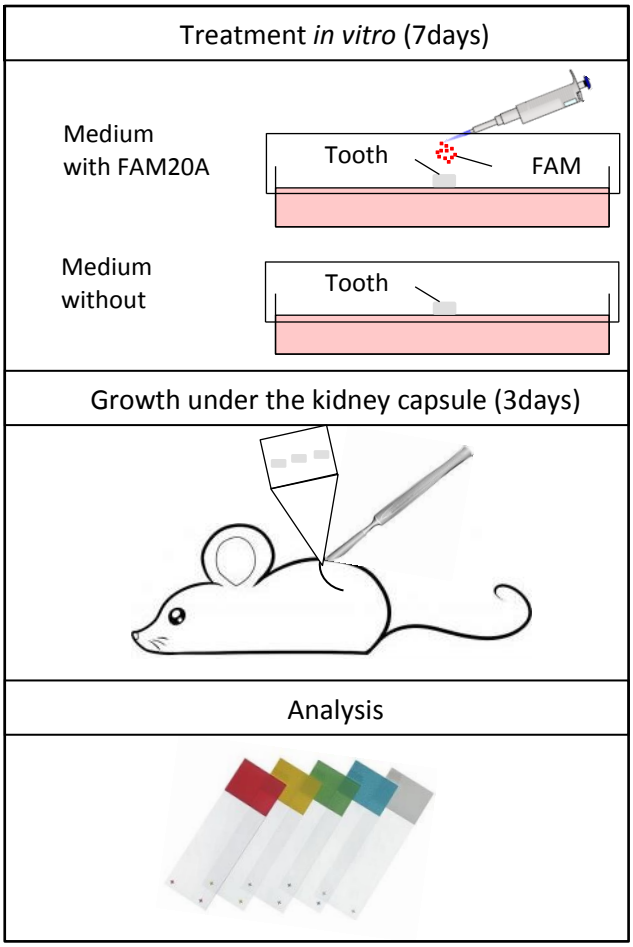


Figure 6.35. Scheme of the phenotype rescue experiment.

Our results show, that when *Fam20a*$^{-/-}$ mouse teeth are treated with the medium containing FAM20A protein, the protein penetrates in the tooth and localizes mostly in odontoblasts and, at a lesser extent, in ameloblasts (Fig. 6.36, 6.37). *Fam20a*$^{-/-}$ mouse teeth that did not receive FAM20A protein were negative for anti-FAM20A IHC staining (Fig. 6.37).

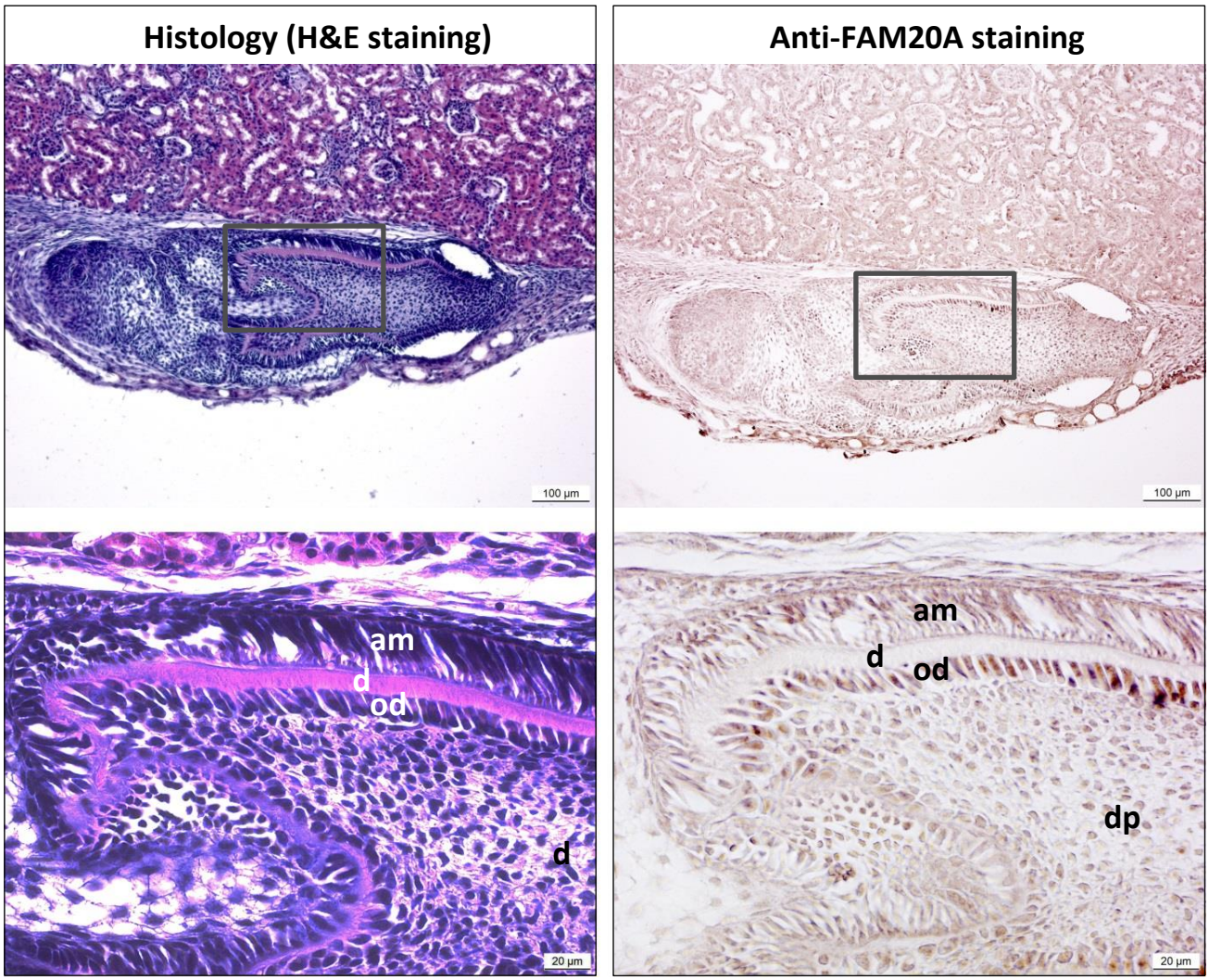


Figure 6.36. Histological analysis and FAM20A protein localization in the *Fam20a*$^{-/-}$ mouse molar after the treatment with the recombinant FAM20A protein. Abbreviations: am – ameloblasts, d – dentin, dp – dental pulp, od – odontoblasts.

Both molars and incisors of *Fam20a*$^{-/-}$ mice that received FAM20A treatment form an organized layer of polarized ameloblasts (Fig. 6.36, 6.38). At the stage of analysis the layer of dentin is already forming, while enamel is just starting to be secreted. To make a conclusion if enamel defect is rescued teeth have to be left longer to grow till the stage of active enamel formation.

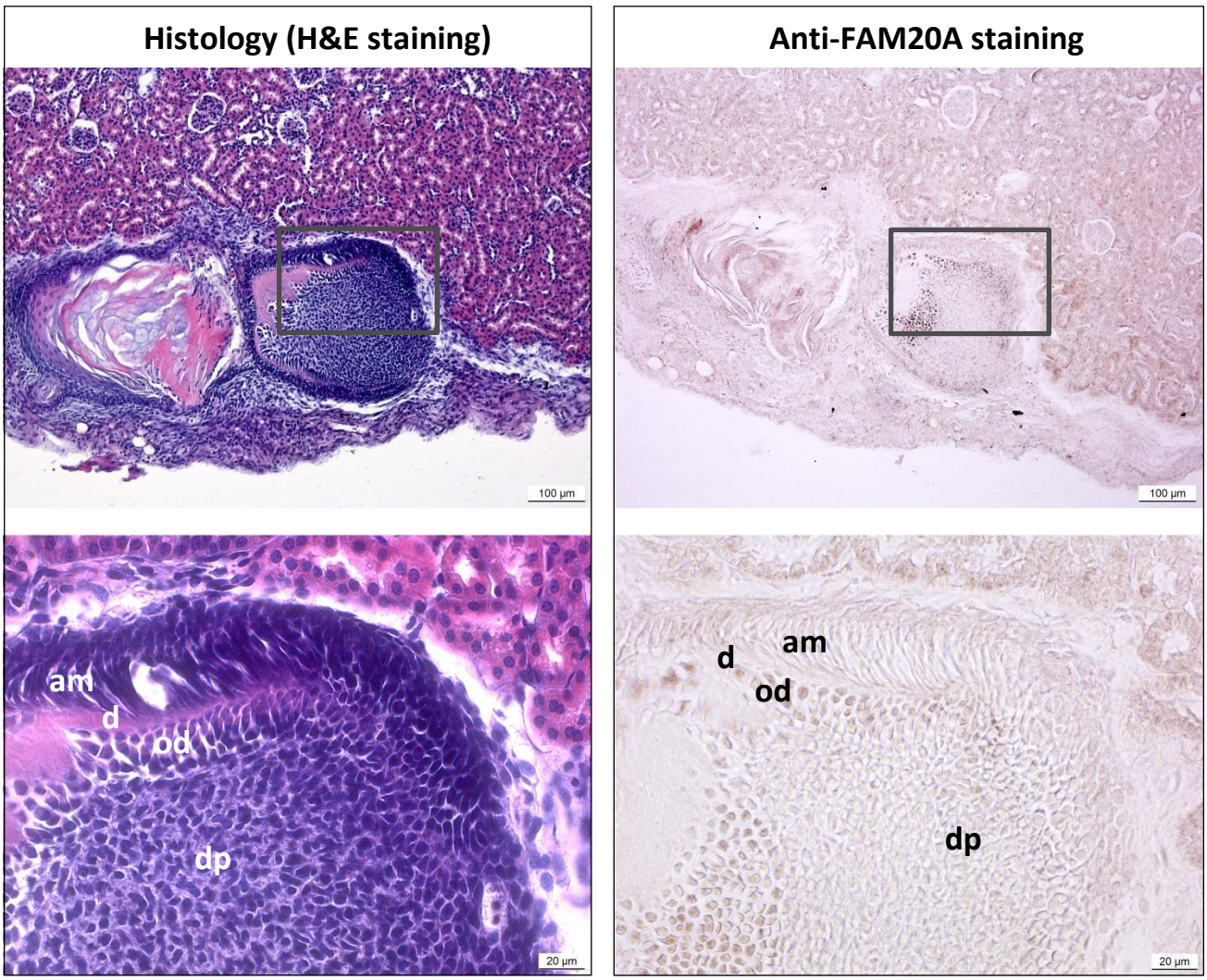


Figure 6.37. Histological analysis and FAM20A protein localization in the *Fam20a*$^{-/-}$ mouse molar without the treatment with the recombinant FAM20A protein. Abbreviations: am – ameloblasts, d – dentin, dp – dental pulp, od – odontoblasts.

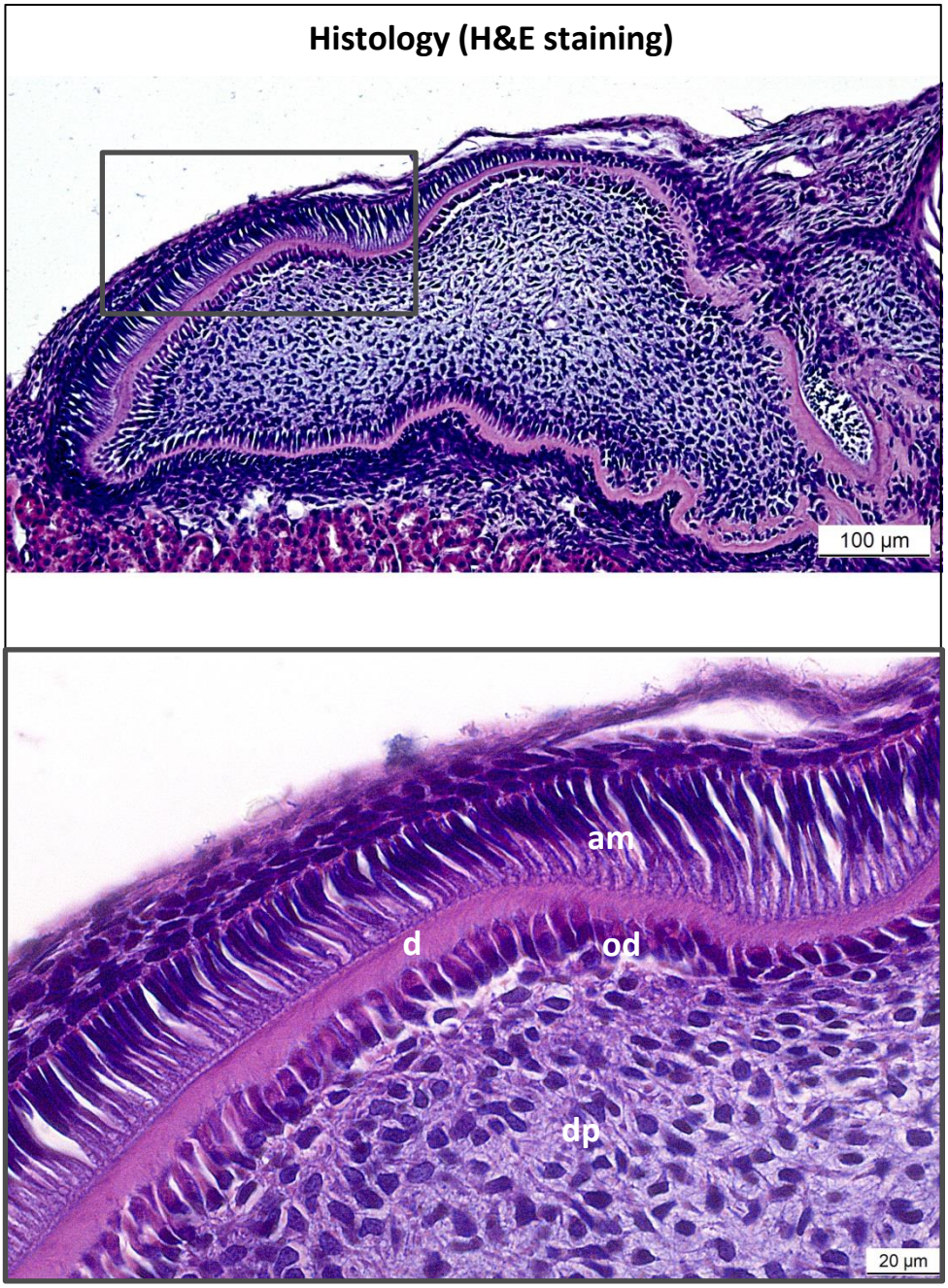


Figure 6.38. Histological analysis of the *Fam20a*$^{-/-}$ mouse incisor after the treatment with the recombinant FAM20A protein. Abbreviations: am – ameloblasts, d – dentin, dp – dental pulp, od – odontoblasts.

7. Discussion

An increasing number of seminal articles published the last few years have demonstrated the importance of members of the new family of Fam20 molecules in regulating biomineralization processes (Vincent S Tagliabracci et al. 2013; Tagliabracci et al. 2015). However, the exact mechanisms of their action and roles in the various organs and tissues are still unknown. Here we report on the expression, regulation, and roles of *Fam20a* in tooth development and pathology.

A study realized this year has shown that Fam20a potentiates the activity of the Fam20c kinase that is crucial for the phosphorylation of the tooth specific enamel proteins (Cui et al. 2015). Therefore, it is of interest to compare the expression patterns of the Fam20a and Fam20c proteins during odontogenesis and discuss defects that have been observed in both *Fam20a* and *Fam20c* mutants.

Overlapping expression patterns of Fam20a and Fam20c in epithelial tissues during odontogenesis

Tooth development and most particularly amelogenesis depends on epithelial – mesenchymal interactions. (Mitsiadis & Graf 2009; Jussila & Thesleff 2015). During embryonic development, expression of the *Fam20a* gene and the Fam20a protein is detected both in epithelial and mesenchymal cells of the tooth. This expression pattern is similar to the expression of the *Fam20c* gene and its product in dental tissues (Wang et al. 2010). At postnatal stages, Fam20a protein is detected mainly in ameloblasts and in a lesser degree in odontoblasts. It has been reported that Fam20c protein is expressed at the same level in odontoblasts and ameloblasts. These expression patterns are indicative of the dental phenotypes observed in both *Fam20a* and *Fam20c* mutants. However, the defects in the *Fam20a* mutants are present exclusively in the enamel, while in the *Fam20c* mutants both enamel and dentin structures are affected (Vogel et al. 2012; Wang et al. 2015; Liu et al. 2014). The overlapping expression pattern of Fam20a and Fam20c, the enamel defects in both mutants, and the biochemical studies on the phosphorylation suggest that these two molecules may act together *in vivo* and are necessary for enamel formation.

Based on our results, the expression of *Fam20a* gene also overlapps with that of the *Amg* and *Amb* genes in developing teeth and more specifically in differentiating and mature ameloblasts. Fam20a protein is also co-expressed with the Amg and Amb proteins in ameloblasts (Nakamura et al. 1994; Snead et al. 1988; Torres-Quintana et al. 2005). Moreover, expression of Fam20a overlaps with that of the *Dspp* both in ameloblasts and in odontoblasts.

Similar enamel defects in Fam20a and Fam20c mouse mutants

Mutations in *Fam20a* and *Fam20c* lead to similar dental phenotypes where the enamel is seriously affected. In the *Fam20a* mutants, the ameloblasts detach from the dentin at their early secretory stage, lose the columnar shape and the organization. The matrix produced by these disorganized ameloblasts is very thin and only partly attached to the dentin, same as in *Fam20c*$^{-/-}$ mice (X. Wang et al. 2012; X. Wang et al. 2013). Since Fam20a does not contain a kinase activity (Cui et al. 2015), it cannot substitute for Fam20c in the ameloblasts of the *Fam20c*$^{-/-}$ mutants. Taken together, these observations reinforce our hypothesis that Fam20a act specifically in the various stages of amelogenesis and is one important molecule for the formation of enamel matrix.

The differential expression pattern of Fam20a and Fam20c in odontoblasts and the absence of dentin defects in the *Fam20a* mutants brings to the assumption that Fam20c kinase works through a different mechanism for the formation of dentin.

Role of Fam20a in enamel development

Fam20a is expressed in both epithelial and mesenchymal dental components thus suggesting that tissue interactions might affect *Fam20a* expression and function in dental epithelium.

Classical early tissue recombination experiments showed that the mesenchyme contains the odontogenic potential and that the epithelium is responding to mesenchymal-derived signal after E12. This applies even when dental mesenchyme is recombined with non-dental epithelium such as epithelium of plantar surface of the foot plate, epithelium from the snout, and lip-furrow epithelium and epithelium from toothless species, such as chick oral epithelium. In all cases well structured teeth were formed, while recombination vice virsa did not lead to the formation of teeth (Kollar & Baird 1970; Kollar 1980).

We recombinaned epithelium from *Fam20a* mutant teeth with mesenchyme from wild type teeth and vice versa. Recombination of *Fam20a*$^{-/-}$ dental epithelium and mesenchyme from wild type teeth gave rise to teeth with defective enamel that strongly resembles to the teeth of *Fam20a*$^{-/-}$ mice. The recombination of dental epithelium from wild type mice and *Fam20a*$^{-/-}$ dental mesenchyme gave rise to teeth with normal enamel. These experiments show that Fam20a expression in epithelium is not influenced by epithelial – mesenchymal interaction during the late stages of amelogenesis.

The role of Fam20c in enamel formation using *K14-Cre;Fam20Cfl/fl* mice where the deletion of Fam20c will be restricted to dental epithelial cells. These transgenic mice exhibited defective enamel (X. Wang et al. 2013). In contrast, *Wnt1-Cre;Fam20Cfl/fl* mice exhibited only defective dentin, since *Wnt1* expression is specific for mesenchymal cells that are involved in the formation of dentin (Wang et al. 2015).

Taken together, these results show that epithelial expression of Fam20a and Fam20c is sufficient for correct enamel development.

Rescuing the enamel phenotype in Fam20a mutant

Since wild type mesenchyme cannot rescue the phenotype in *Fam20a*$^{-/-}$ epithelium, we attempt to rescue the function of Fam20a in the tooth of *Fam20a*$^{-/-}$ mice by the addition of a recombinant FAM20A protein. We have shown that the protein is able to penetrate in the developing tooth germ and is specifically localized in odontoblasts and ameloblasts, with the pattern similar to *in vivo* conditions. After addition of the protein, ameloblasts appeared normal, well organized and fully polarized. Further experiments will aim to study if the enamel formation and structure is also rescued. Optimal doses of the protein and other ways of in vivo delivery are under investigation.

Several attemps were made to rescue defects in dentin and bone of *Fam20c*$^{-/-}$ mice (Wang et al. 2014). These defects are very similar to the phenotype of *Dmp1* mouse mutants (Feng et al. 2006). Additionally, Dmp1 is significantly downregulated in *Fam20c* conditional knockout mice (Xiaofang Wang, Wang, Li, et al. 2012; Xiaofang Wang, Wang, Lu, et al. 2012) and it belongs to the SIBLINGs family of proteins, which are phosphorylated by Fam20c (Ishikawa et al. 2012; Tagliabracci, Engel, Wen, Wiley, C. A. Worby, et al. 2012). Taken together, these observations suggest that overexpression of Dmp1 in mineralized tissues in Fam20c mutants may rescue the bone and dentin phenotype. However, expressing the Dmp1 transgene failed to repair the dental defects (Wang et al. 2014).

Phosphorylation events induced by Fam20c-Fam20a

It is not known if phosphorylation of the proteins by Fam20a-Fam20c complex takes place intracellularly or extracellularly. Since Fam20a function in enamel development depends on the presence of Fam20c, it is of interest to compare the exact localization of those two molecules.

Our results show that Fam20a protein is localized in both secretory ameloblasts and enamel matrix. However, previous results have shown that Fam20c is localized only intracellularly. The Fam20c protein was detected only in ameloblasts, but not in the enamel matrix (S.-K. Wang et al. 2013). Some contradictory data suggest that Fam20c is a secreted protein: *Fam20c* gene has a signal peptide sequence (Nalbant et al. 2005), Fam20c was found in the bovine milk, and ectopically expressed Fam20c is secreted (Tagliabracci, Engel, Wen, Wiley, C. a Worby, et al. 2012).

Even though Fam20a and Fam20c are secreted proteins, there is no evidence that phosphorylation events occur extracellularly (Vincent S. Tagliabracci et al. 2013).

Role of Fam20a

Based on the phenotype observed in *Fam20a* mutants, Fam20a might have an effect not only on the phosphorylation of enamel proteins, but may affect the function of other molecules, including cell adhesion molecules (Fig. 8.1). It could also happen that disorganization of the ameloblastic layer is a consequnce of the wrong function of enamel proteins.

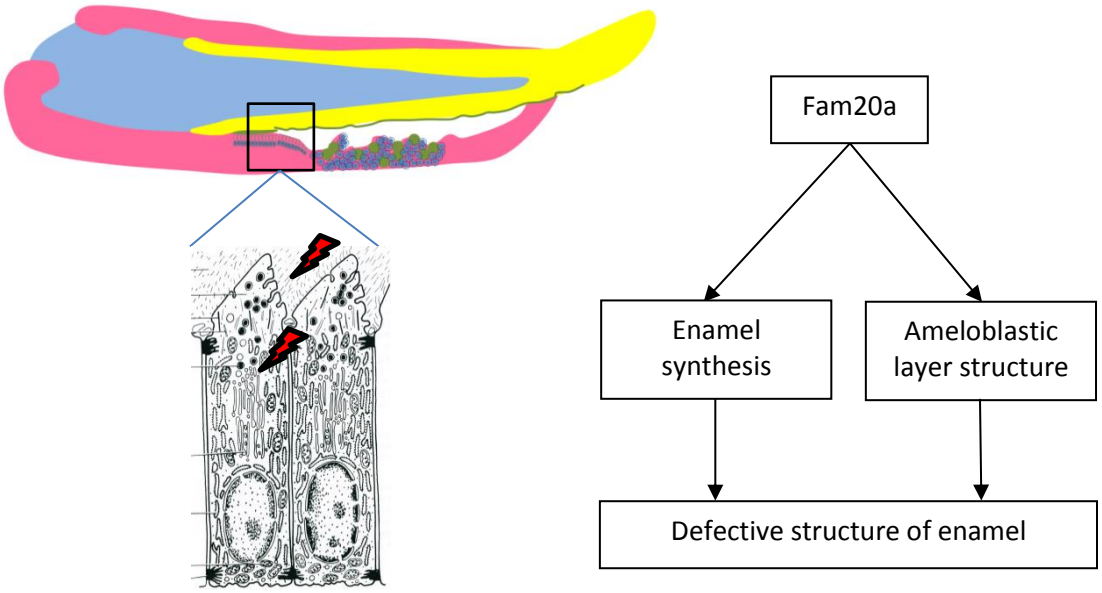


Figure 8.1. *Role of Fam20a in enamel development and pathology.*

The enamel proteins Amg, Amb and Enam have already been shown to be phosphorylated by the Fam20c kinase (Vincent S Tagliabracci et al. 2013). Post-translational modifications are crucial for proper function proteins (Walsh et al. 2005). Thus, mutations in the enamel proteins probably lead to the same type of enamel defects as the loss of posttranslational modification. Mutations of *Amb* lead to the same enamel defects as mutations in *Fam20a* and *Fam20c*, but this is not the case in mutations affecting *Enam* or *Amg* (Wazen et al. 2009; Hu et al. 2008; Wright et al. 2008). While we did not observe any change in *Amb* gene expression in Fam20a mouse mutant, *Amb* and *Ambt* were shown to be expressed at the lower levels in the Fam20c mutants than in wild type mice (X. Wang et al. 2013).

This topic is important, since the alteration of kinase activity is already used to treat inflammatory and autoimmune diseases, hypertension, Parkinson's disease, rheumatoid arthritis, myelofibrosis and cancer. In the last 15 years, the pharmaceutical industry in the field of cancer has chosen protein kinases as their main target (Cohen & Alessi 2014; Held 2012). Our research adds value by providing new data on the roles of protein kinases in mineralization processes. Moreover, it opens perspectives for the discovery of new treatments for the Fam20a-associated pathologies.

8. Abbreviations

AI: amelogenesis imperfecta

Amb: ameloblastin

Amg: amelogenin

Amtn: amelotin

ATP: adenosine triphosphate

BL: basal lamina

BM: basal membrane

Bmp: bone morphogenetic protein

Bsp: bone sialoprotein

Ca: calcium

CL: cervical loop

Cre: cre recombinase

Dmp1: dentin matrix protein 1

Dspp: dentin sialophosphoprotein

Eda: ectodysplasin

EDTA: ethylenediaminetetraacetic acid

Enam: enamelin

Fam20: family with sequence similarity 20

Fgf: fibroblast growth factor

GA: glycosaminoglycan

IHC: immunohistochemistry

ISH: in situ hybridisation

K14: keratin 14

Klk4: kallikrein-related peptidase 4

Lef1: gene for lymphoid enhancer-binding factor 1

Mg: magnesium

Mmp20: matrix metalloproteinase 20

Msx1: msh homeobox 1

Odam: odontogenic ameloblast-associated

Osp: osteopontin

Osc: osteocalcin

Osn: osteonectin

P: phosphorus

Pax: paired box gene

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Pitx: paired-like homeodomain transcription factor

PDL: periodontal ligament

PFA: paraformaldehyde

PG: proteoglycan

S: sulfur

Scpppq1: secretory calcium-binding phosphoprotein-proline-glutamine-rich 1

Ser: serin

Shh: sonic hedgehog

SIBLING: small integrin-binding ligand, N-linked glycoprotein

Sox2: SRY (sex determining region Y)-box 2

SSC: Saline Sodium Citrate Stock Solution

Thr: threonin

Tyr: tyrosin

wt: wild type

9. Curriculum vitae

Personal information:

Name:	Anna Filatova
Date of birth:	16.04.1989
Place of birth:	Barnaul, Russia
Address:	Dübendorfstrasse 34, 8051, Zürich, Switzerland
Phone:	+41 78 915 61 03
	+41 (0)44 634 31 97 (Office)
E-mail:	annafilatova89@gmail.com, anna.filatova@zzm.uzh.ch

Education:

12/11 – now **University of Zurich**
PhD student in Molecular Life Science program

09/06 – 07/11 **Novosibirsk State University**
Bachelor and Master student, Department of Natural Sciences, Novosibirsk, Russia

09/05 – 06/06 **Novosibirsk State University**
Physical-Mathematical school under university, Novosibirsk, Russia

09/96 – 06/05 **Gymnasium** 42
Barnaul, Russia

Practice and professional experience:

12/11 – now **University of Zurich**, Institute of Oral Biology, Center of Dental Medicine, Zurich, Switzerland

Position: PhD student

- Clinical oriented research
- Various clinical and biological conferences and schools
- Working in multidisciplinary and international teams
- Presenting projects at different meetings and events
- Multitasking and working at several projects
- Teaching

06/14 – now **Paul Scherrer Institute**, Villigen, Switzerland

Position: project in collaboration

- Biochemical techniques

08/14 – now **University of Zurich**, Molecular Life Science Program, Zurich, Switzerland

Position: member of lecture organising committee

- inviting world recognised scientists
- planning the budget
- organising the whole event

- preparing leaflets
- advertising the event

(flights, meetings, lectures, dinner)

02/09 – 07/11 **Siberian Branch of Russian Academy of Sciences**, Laboratory of Developmental Epigenetics, Institute of Cytology and Genetics, Novosibirsk, Russia

Position: undergraduate student

Languages

Russian	Mother tongue
English	Fluent
German	Level B1/B2

IT-Skills

Data managing	MS Office, Outlook
Graphic programs	Adobe Photoshop

Honours

04/11	**Best presentation award** for International student, postgraduate and young scientist conference "Lomonosov-2011", subsection "Genetics", Moscow, Russia
11	Potanin's **Scholarship for Extraordinary students** (http://www.stipendia.ru/)
06/10-06/11	**Increased University Fellowship**

Conferences and summer schools

2015	Summer school in Translational Biology, Interlaken, Switzerland
2015	Swiss stem cell network, Basel, Switzerland
2014	RICA 2014 conference "Sustainable Supply Chains", Zurich, Switzerland
2014	10th annual meeting of "Swiss Stem Cell Network", Geneva, Switzerland
2014	13th day of clinical research, Zurich, Switzerland
2013	International conference "Tooth Morphogenesis and Differentiation", La Londe les Maures, France
2013	12th day of clinical research, Zurich, Switzerland
2013	9th annual meeting of "Swiss stem cell network", Bern, Switzerland
2012	74th Annual Meeting of the Swiss Society for Anatomy, Histology, and Embryology "Building Tissues: morphogenesis, regeneration, and replacement", University Zurich, , Switzerland
2012	Annual Retreat of Konstanz Research School Chemical Biology, Gültstein, Germany
2012	Annual Retreat of Molecular Life Science Program, University of Zurich, , Switzerland

2011 International student, postgraduate and young scientist conference “Lomonosov-2011”, Moscow, Russia

Publications

2014 „EMMPRIN7CD147 deficiency disturbs ameloblast-odontoblast cross-talk and delays enamel mineralisation“, Bone

2014 „Distribution of the amelogenin protein in developing, injured and carious human teeth“, Frontiers in Physiology / Craniofacial biology

2014 „Distribution of syndecan-1 protein in developing mouse teeth“, Frontiers in Physiology, section Craniofacial Biology

2013 „Regenerative Dentistry: Stem Cells meet Nanotechnology“, in book: Horizons in Clinical Nanomedicine, Edition: 1st edition, Editors: Pan Stanford Publishing

10. Acknowledgments

First of all I would like to thank my supervisor Prof. Thimios Mitsiadis, who supported and challenged my growth as a scientist. He gave me an opportunity to work on several very exciting projects and also be a part of interesting collaborations. I am very grateful for all his guidance and also that he let me learn how to be independent. He taught me how to be creative in asking scientific questions and finding solutions, how to find your own place in a very competitive and changing world of science. He always supported my scientific interests and let me attend interesting conferences and schools, for this I would also like to thank him.

I am very grateful to the members of my committee: Prof. Michael Hengartner, Prof. Sabine Werner, Prof. Freddy Radtke and Prof. Alex Hajnal for their critical vision of my project, for questions that were discussed and ideas that they gave. Meetings and discussions with the committee members made a big influence on me as a scientist.

I would like to thank our collaborators Prof. Hans Ullrich-Luder and collaborators from Paul Scherrer Institute (PSI) Prof. Kurt Ballmer-Hofner and Dr. Maria Mitsi. I spend almost a year in PSI, learning new techniques and new scientific approach. I was treated as a group member and I am very thankful for this experince. And I am also very grateful for the recombinant protein that we produced together.

Many thanks go to my lab collegues with whom I spend almost every day of my life during last 4 years. To Dr. Lucia Jimenez for supervising me at first steps and giving a lot of help and support with experiments afterwards. To Dr. Pagella for always being very helpful with experiments and animals. To Anna Woloszyk who helped for the critical reading. To Despoina Natsiou with whom we shared a laboratory. Also many thanks to Christina Lämmler for her help with any problems at work and outside work. Also to Angela Di Pietro who was very supportive in critical moments. To Jinan who helped with every day life in the lab. To other members of the institute who is still here and who has left the lab.

11. References

Alachioti, X.S. et al., 2014. Amelogenesis imperfecta and anterior open bite: Etiological, classification, clinical and management interrelationships. *Journal of orthodontic science*, 3(1), pp.1–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4072389&tool=pmcentrez&rendertype=abstract.

Aldred, M.J., Savarirayan, R. & Crawford, P.J.M., 2003. Amelogenesis imperfecta: a classification and catalogue for the 21st century. *Oral diseases*, 9(1), pp.19–23.

Ang, S.F. et al., 2012. Comparison of mechanical behaviors of enamel rod and interrod regions in enamel. *Journal of Materials Research*, 27(02), pp.448–456.

Bartlett, J.D., 2013a. Dental enamel development: proteinases and their enamel matrix substrates. *ISRN dentistry*, 2013, p.684607.

Bartlett, J.D., 2013b. Dental enamel development: proteinases and their enamel matrix substrates. *ISRN dentistry*, 2013, p.684607. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3789414&tool=pmcentrez&rendertype=abstract.

Cabral, R.M. et al., 2013. Autosomal recessive gingival hyperplasia and dental anomalies caused by a 29-base pair duplication in the FAM20A gene. *Journal of human genetics*, 58(8), pp.566–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23697977 [Accessed February 12, 2015].

Catón, J. et al., 2011. Future dentistry: Cell therapy meets tooth and periodontal repair and regeneration. *Journal of Cellular and Molecular Medicine*, 15(5), pp.1054–1065.

Cherkaoui Jaouad, I. et al., 2015. Further evidence for causal FAM20A mutations and first case of amelogenesis imperfecta and gingival hyperplasia syndrome in Morocco: a case report. *BMC oral health*, 15(1), p.14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25636655 [Accessed February 19, 2015].

Cho, S.H. et al., 2012. Novel FAM20A mutations in hypoplastic amelogenesis imperfecta. *Human mutation*, 33(1), pp.91–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21990045 [Accessed February 12, 2015].

Cobourne, M.T. & Sharpe, P.T., 2010. Making up the numbers: The molecular control of mammalian dental formula. *Seminars in Cell and Developmental Biology*, 21(3), pp.314–324.

Cohen, P. & Alessi, D.R., 2014. Europe PMC Funders Group Kinase Drug Discovery – What ' s Next in the Field ? , 8(1), pp.96–104.

Crawford, P.J.M., Aldred, M. & Bloch-Zupan, A., 2007. Amelogenesis imperfecta. *Orphanet journal of rare diseases*, 2, p.17.

Cui, J. et al., 2015. A secretory kinase complex regulates extracellular protein phosphorylation. *eLife*, 4, p.e06120. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4421793&tool=pmcentrez&rendertype=abstract [Accessed August 3, 2015].

Diep, L. et al., 2009. Contribution of the tooth bud mesenchyme to alveolar bone. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 312(5), pp.510–517.

Donoghue, P.C.J. & Rücklin, M., 2014. The ins and outs of the evolutionary origin of teeth. *Evolution & Development*, 12, p.n/a–n/a.

Eames, B.F. et al., 2011. Mutations in fam20b and xylt1 reveal that cartilage matrix controls timing of endochondral ossification by inhibiting chondrocyte maturation. *PLoS genetics*, 7(8), p.e1002246. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3161922&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Faundes, V. et al., 2014. Raine syndrome: an overview. *European journal of medical genetics*, 57(9), pp.536–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25019372 [Accessed February 25, 2015].

Feng, J.Q. et al., 2006. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nature genetics*, 38(11), pp.1310–5. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-33750454816&partnerID=tZOtx3y1 [Accessed October 7, 2015].

Filatova, A., Pagella, P. & Mitsiadis, T. a, 2014. Distribution of syndecan-1 protein in developing mouse teeth. *Frontiers in physiology*, 5(January), p.518. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4295547&tool=pmcentrez&rendertype=abstract [Accessed August 3, 2015].

Fleischmannova, J. et al., 2010. Formation of the tooth-bone interface. *Journal of dental research*, 89(2), pp.108–115.

Fukumoto, S. et al., 2004. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. *Journal of Cell Biology*, 167(5), pp.973–983.

Gibson, C.W. et al., 2001. Amelogenin-deficient Mice Display an Amelogenesis Imperfecta Phenotype. *Journal of Biological Chemistry*, 276(34), pp.31871–31875.

Gordon, L.M. & Joester, D., 2015. Mapping residual organics and carbonate at grain boundaries and the amorphous interphase in mouse incisor enamel. *Frontiers in Physiology*, 6(March), pp.1–10. Available at: http://www.frontiersin.org/Craniofacial_Biology/10.3389/fphys.2015.00057/abstract.

Held, D., 2012. (P ng K / A ml) (P ng K / A ml). , 1(19).

Hormia, M., Owaribe, K. & Virtanen, I., 2001. The dento-epithelial junction: cell adhesion by type I hemidesmosomes in the absence of a true basal lamina. *The Journal of periodontology*, 72(6), pp.788–797.

Hu, J.C.C. et al., 2008. Enamel defects and ameloblast-specific expression in Enam knock-out/lacZ knock-in mice. *Journal of Biological Chemistry*, 283(16), pp.10858–10871.

Hu, J.C.C. et al., 2007. Enamel formation and amelogenesis imperfecta. *Cells Tissues Organs*, 186(1), pp.78–85.

Hu, J.C.C. et al., 2014. Enamelin is critical for ameloblast integrity and enamel ultrastructure formation. *PLoS ONE*, 9(3), pp.1–13.

Hu, J.C.C. et al., 2002. Enamelysin and kallikrein-4 mRNA expression in developing mouse molars. *European journal of oral sciences*, 110(4), pp.307–315.

Ishikawa, H.O. et al., 2012. The Raine syndrome protein FAM20C is a Golgi kinase that phosphorylates bio-mineralization proteins. *PloS one*, 7(8), p.e42988. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3416761&tool=pmcentrez&rendertype=abstract [Accessed February 17, 2015].

Jaureguiberry, G. et al., 2012. Nephrocalcinosis (enamel renal syndrome) caused by autosomal recessive FAM20A mutations. *Nephron. Physiology*, 122(1-2), pp.1–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3782194&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Jussila, M. & Thesleff, I., 2015. Signaling Networks Regulating Tooth Organogenesis and Regeneration , and the Specification of Dental Mesenchymal and Epithelial Cell Lineages.

Kantaputra, P.N. et al., 2014. Enamel-Renal-Gingival syndrome, hypodontia, and a novel FAM20A mutation. *American journal of medical genetics. Part A*, 164A(8), pp.2124–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24756937 [Accessed February 12, 2015].

Kim, J.-W. et al., 2006. Mutational analysis of candidate genes in 24 amelogenesis imperfecta families. *European journal of oral sciences*, 114 Suppl (10), pp.3–12; discussion 39–41, 379.

Koike, T. et al., 2009. FAM20B is a kinase that phosphorylates xylose in the glycosaminoglycan-protein linkage region. *The Biochemical journal*, 421(2), pp.157–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19473117 [Accessed February 18, 2015].

Kollar, E.J. & Baird, G.R., 1970. Tissue interactions in embryonic mouse tooth germs. II. The inductive role of the dental papilla. *Journal of embryology and experimental morphology*, 24(1), pp.173–186.

Kollar, E.J.. F.C., 1980. Tooth Induction in Chick Epithelium: expression of quiescent genes for enamel synthesis. *Science*, 207(February 1980), pp.993–995.

De la Dure-Molla, M. et al., 2014. Pathognomonic oral profile of Enamel Renal Syndrome (ERS) caused by recessive FAM20A mutations. *Orphanet journal of rare diseases*, 9(1), p.84. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4071802&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Lacruz, R.S. et al., 2012. New paradigms on the transport functions of maturation-stage ameloblasts. *Journal of dental research*, 92(2), pp.122–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3545694&tool=pmcentrez&rendertype=abstract.

Lacruz, R.S. et al., 2010. Regulation of pH during amelogenesis. *Calcified Tissue International*, 86(2), pp.91–103.

Liu, P. et al., 2014. Inactivation of Fam20C in cells expressing type I collagen causes periodontal disease in mice. *PloS one*, 9(12), p.e114396. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4257665&tool=pmcentrez&rendertype=abstract [Accessed February 17, 2015].

Lokappa, S.B., Chandrababu, K.B. & Moradian-Oldak, J., 2015. Tooth enamel protein amelogenin binds to ameloblast cell membrane-mimicking vesicles via its N-terminus. *Biochemical and Biophysical Research Communications*, pp.1–6. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0006291X15303120.

Martelli-Júnior, H. et al., 2008. Case reports of a new syndrome associating gingival fibromatosis and dental abnormalities in a consanguineous family. *Journal of periodontology*, 79(7), pp.1287–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18597613 [Accessed February 19, 2015].

Masuya, H. et al., 2005. Enamelin (Enam) is essential for amelogenesis: ENU-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (AI). *Human Molecular Genetics*, 14(5), pp.575–583.

Matalova, E. et al., 2005. Cell lineage of primary and secondary enamel knots. *Developmental Dynamics*, 233(3), pp.754–759.

Mddica, D.G., 1988. Unknown syndrome : , pp.786–789.

Mitsiadis, T. a et al., 2011. Dental pulp stem cells, niches, and notch signaling in tooth injury. *Advances in dental research*, 23(3), pp.275–279.

Mitsiadis, T. a et al., 2014. Distribution of the amelogenin protein in developing, injured and carious human teeth. *Frontiers in physiology*, 5(December), p.477. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4261713&tool=pmcentrez&rendertype=abstract [Accessed July 22, 2015].

Mitsiadis, T. a. & Graf, D., 2009. Cell fate determination during tooth development and regeneration. *Birth Defects Research Part C - Embryo Today: Reviews*, 87(3), pp.199–211.

Mitsiadis, T. & Luder, H., 2011. Genetic basis for tooth malformations: From mice to men and back again. *Clinical Genetics*, 80(4), pp.319–329.

Mitsiadis, T.A. et al., 1995. Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development*, 121(1), pp.37–51.

Moffatt, P. et al., 2014. Characterisation of secretory calcium-binding phosphoprotein-proline-glutamine-rich 1: a novel basal lamina component expressed at cell-tooth interfaces. *Cell and Tissue Research*, 358(3), pp.843–855. Available at: http://link.springer.com/10.1007/s00441-014-1989-3.

Nadanaka, S. et al., 2013. EXTL2, a member of the EXT family of tumor suppressors, controls glycosaminoglycan biosynthesis in a xylose kinase-dependent manner. *The Journal of biological chemistry*, 288(13), pp.9321–33. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3611003&tool=pmcentrez&rendertype=abstract [Accessed February 18, 2015].

Nadanaka, S. & Kitagawa, H., 2014. EXTL2 controls liver regeneration and aortic calcification through xylose kinase-dependent regulation of glycosaminoglycan biosynthesis. *Matrix biology : journal of the International Society for Matrix Biology*, 35, pp.18–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24176719 [Accessed February 18, 2015].

Nakamura, M. et al., 1994. Translocation of enamel proteins from inner enamel epithelia to odontoblasts during mouse tooth development. *The Anatomical record*, 238(3), pp.383–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8179220.

Nakayama, Y., Holcroft, J. & Ganss, B., 2015. Enamel Hypomineralization and Structural Defects in Amelotin-deficient Mice. *Journal of dental research*, 94(5), pp.697–705. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25715379 [Accessed August 3, 2015].

Nalbant, D. et al., 2005. FAM20: an evolutionarily conserved family of secreted proteins expressed in hematopoietic cells. *BMC genomics*, 6, p.11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=548683&tool=pmcentrez&rendertype=abstract [Accessed February 16, 2015].

Nanci, A., 2003. *Ten Cate's Oral Histology* 6th ed., Mosby.

O'Sullivan, J. et al., 2011. Whole-Exome sequencing identifies FAM20A mutations as a cause of amelogenesis imperfecta and gingival hyperplasia syndrome. *American journal of human genetics*, 88(5), pp.616–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3146735&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Ozdemir, D. et al., 2005. is Dosage-dependent. , 84(11).

Pagella, P., Jiménez-Rojo, L. & Mitsiadis, T. a, 2014. Roles of innervation in developing and regenerating orofacial tissues. *Cellular and molecular life sciences : CMLS*, 71(12), pp.2241–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24395053 [Accessed August 3, 2015].

Perdigão, P.F. et al., 2004. Ameloblastin gene (AMBN) mutations associated with epithelial odontogenic tumors. *Oral Oncology*, 40(8), pp.841–846.

Perdigão, P.F. et al., 2009. Mutation of ameloblastin gene in calcifying epithelial odontogenic tumor. *Anticancer Research*, 29(8), pp.3065–3067.

Pugach, M.K. et al., 2010. The amelogenin C-terminus is required for enamel development. *Journal of dental research*, 89(2), pp.165–169.

Rafaelsen, S.H. et al., 2013. Exome sequencing reveals FAM20c mutations associated with fibroblast growth factor 23-related hypophosphatemia, dental anomalies, and ectopic calcification. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*, 28(6), pp.1378–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23325605 [Accessed February 12, 2015].

Ravindran, S. & George, A., 2015. Biomimetic extracellular matrix mediated somatic stem cell differentiation: applications in dental pulp tissue regeneration. *Frontiers in Physiology*, 6(April), pp.1–9. Available at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00118/abstract.

Dos Santos Neves, J. et al., 2012. Odontogenic ameloblast-associated and amelotin are novel basal lamina components. *Histochemistry and Cell Biology*, 137(3), pp.329–338.

Sasaki, S. & Shimokawa, H., 1995. The amelogenin gene. *International Journal of Developmental Biology*, 39, pp.127–133.

Seymen, F. et al., 2015. Novel MMP20 and KLK4 Mutations in Amelogenesis Imperfecta. *Journal of Dental Research*, 94(8), pp.1063–1069. Available at: http://jdr.sagepub.com/cgi/doi/10.1177/0022034515590569.

Simmer, J.P. et al., 2012. A post-classical theory of enamel biomineralization… and why we need one. *International Journal of Oral Science*, 4(3), pp.129–134.

Simpson, M. a et al., 2009. Mutations in FAM20C also identified in non-lethal osteosclerotic bone dysplasia. *Clinical genetics*, 75(3), pp.271–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19250384 [Accessed February 25, 2015].

Smith, C.E. & Chun, Y.P., 2015. Correlation of ameloblastin with enamel mineral content. , 55(0 1), pp.38–42.

Snead, M.L. et al., 1988. Spatial- and temporal-restricted pattern for amelogenin gene expression during mouse molar tooth organogenesis. , 85, pp.77–85.

Tagliabracci, V.S. et al., 2015a. A Single Kinase Generates the Majority of the Secreted Phosphoproteome. *Cell*, 161(7), pp.1619–1632.

Tagliabracci, V.S. et al., 2015b. A Single Kinase Generates the Majority of the Secreted Phosphoproteome. *Cell*, 161(7), pp.1619–1632. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0092867415005784.

Tagliabracci, V.S., Engel, J.L., Wen, J., Wiley, S.E., Worby, C.A., et al., 2012. Secreted Kinase Phosphorylates Extracellular Proteins that Regulate Biomineralization. , 2(May), pp.2–5.

Tagliabracci, V.S., Engel, J.L., Wen, J., Wiley, S.E., Worby, C. a, et al., 2012. Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science (New York, N.Y.)*, 336(6085), pp.1150–3. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3754843&tool=pmcentrez&rendertype=abstract [Accessed January 11, 2015].

Tagliabracci, V.S., Pinna, L. a & Dixon, J.E., 2013. Secreted protein kinases. *Trends in biochemical sciences*, 38(3), pp.121–30. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3582740&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Tagliabracci, V.S., Pinna, L. a. & Dixon, J.E., 2013. Response to Wang et al.: Secreted protein kinases? *Trends in Biochemical Sciences*, 38(9), p.425. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0968000413000984.

Tagliabracci, V.S., Xiao, J. & Dixon, J.E., 2013. Phosphorylation of substrates destined for secretion by the Fam20 kinases. *Biochemical Society transactions*, 41(4), pp.1061–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23863179 [Accessed February 12, 2015].

Takeyari, S. et al., 2014. Hypophosphatemic osteomalacia and bone sclerosis caused by a novel homozygous mutation of the FAM20C gene in an elderly man with a mild variant of Raine syndrome. *Bone*, 67, pp.56–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24982027 [Accessed February 25, 2015].

Torres-Quintana, M.A. et al., 2005. Ameloblastin and amelogenin expression in posnatal developing mouse molars. *Journal of oral science*, 47(1), pp.27–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15881226.

Tucker, A. & Sharpe, P., 2004. The cutting-edge of mammalian development; how the embryo makes teeth. *Nature reviews. Genetics*, 5(7), pp.499–508.

Vogel, P. et al., 2012. Amelogenesis imperfecta and other biomineralization defects in Fam20a and Fam20c null mice. *Veterinary pathology*, 49(6), pp.998–1017. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22732358 [Accessed February 12, 2015].

Walsh, C.T., Garneau-Tsodikova, S. & Gatto, G.J., 2005. Protein Posttranslational Modifications: The Chemistry of Proteome Diversifications. *Angewandte Chemie International Edition*, 44(45), pp.7342–7372. Available at: http://doi.wiley.com/10.1002/anie.200501023 [Accessed July 9, 2014].

Wang, S.K. et al., 2014. FAM20A mutations associated with enamel renal syndrome. *Journal of dental research*, 93(1), pp.42–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3865794&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, S.-K., Aref, P., et al., 2013. FAM20A mutations can cause enamel-renal syndrome (ERS). *PLoS genetics*, 9(2), p.e1003302. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3585120&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, S.-K., Samann, A.C., et al., 2013. FAM20C functions intracellularly within both ameloblasts and odontoblasts in vivo. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*, 28(12), pp.2508–11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3836880&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, X. et al., 2010. Expression of FAM20C in the osteogenesis and odontogenesis of mouse. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 58(11), pp.957–67. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2958138&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, X. et al., 2012. FAM20C Plays an Essential Role in the Formation of Murine Teeth. *Journal of Biological Chemistry*, 287(43), pp.35934–35942. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.M112.386862.

Wang, X., Wang, S., Lu, Y., et al., 2012. FAM20C plays an essential role in the formation of murine teeth. *The Journal of biological chemistry*, 287(43), pp.35934–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3476261&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, X., Wang, S., Li, C., et al., 2012. Inactivation of a novel FGF23 regulator, FAM20C, leads to hypophosphatemic rickets in mice. *PLoS genetics*, 8(5), p.e1002708. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3355082&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Wang, X. et al., 2014. Overexpression of Dmp1 fails to rescue the bone and dentin defects in Fam20C knockout mice. *Connective tissue research*, 55(4), pp.299–303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24874551 [Accessed February 25, 2015].

Wang, X. et al., 2013. The specific role of FAM20C in amelogenesis. *Journal of dental research*, 92(11), pp.995–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3797537&tool=pmcentrez&rendertype=abstract [Accessed February 25, 2015].

Wang, X. et al., 2015. The Specific Role of FAM20C in Dentinogenesis. *Journal of dental research*, 94(2), pp.330–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25515778 [Accessed February 25, 2015].

Wazen, R.M. et al., 2009. A mouse model expressing a truncated form of ameloblastin exhibits dental and junctional epithelium defects. *Matrix biology : journal of the International Society for Matrix Biology*, 28(5), pp.292–303. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2727877&tool=pmcentrez&rendertype=abstract [Accessed February 12, 2015].

Weil, A., 2003. Teeth as tools. , 422(March), p.27708.

Wright, J.T. et al., 2008. Human and mouse enamel phenotypes resulting from mutation or altered expression of AMEL, ENAM, MMP20 and KLK4. *Cells Tissues Organs*, 189(1-4), pp.224–229.

Wright, J.T. et al., 2011. The role of amelogenin during enamel-crystallite growth and organization in vivo. *European Journal of Oral Sciences*, 119(SUPPL.1), pp.65–69.

Zhang, Y.D. et al., 2005. Making a tooth : growth factors , transcription factors , and stem cells. , 15(May), pp.301–316.